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CANCER AND OCCUPATION IN THE NETHERLANDS.

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THIS investigation into the mortality from carcinoma in the Netherlands during the years 1931–35 is based on the mortality-cards placed at our disposal for inspection by the courtesy of the Registrar-General.

In the Netherlands a card for each deceased person is filed with the Registrar-General, which mentions, among other things, the following particulars :

1. Male or female.
2. Married, single, divorced, widow(er).
3. Date of birth.
4. Date of death.
5. Occupation of the deceased.
6. Former occupation of the deceased.
7. Occupation of the head of the family (if the deceased himself had no occupation).
8. Cause of death (stated by the physician to the Registrar of births, deaths and marriages).
9. Cause of death (as stated by the physician under bond of secrecy to the Medical Offices attached to the Registrar-General).
10. Diseases from which the deceased suffered, but which were not the cause of death.

TOTAL NUMBER OF DEATHS FROM CARCINOMA.

The total number of deaths from malignant tumours in the years 1931–35 amounted to 51,124. During this period there were 49,245 deaths from carcinoma, so that the total number of sarcomas, etc., was 1879, or less than 4 per cent of all malignant tumours.

More women than men die from carcinoma (25,927 women to 23,318 men), but on the other hand there are more women over the age of 40 than men. Taking into consideration the difference in number and the different age-grouping between men and women we find a somewhat different ratio. If there had been equal numbers of men and women, and equal numbers in the age-groups, on an average the male mortality would have amounted to 24,855. The number of female

carcinoma deaths (only the deaths over 30 years of age are computed here) was 25,760. The difference is therefore much smaller (905 instead of 2609) than with the uncorrected figures.

TABLE I.—*Incidence of Carcinoma in the Various Organs of Men and Women.*

	Men.		Women.	
	Number.	Percentage of total.	Number.	Percentage of total.
Stomach	10,579	45·37	7559	29·16
Rectum	1,533	6·57	1239	4·78
Oesophagus	1,555	6·67	565	2·18
Intestines	1,747	7·49	2497	9·63
Peritoneum	131	0·56	334	1·29
Tongue	222	0·95	55	0·21
Lip	111	0·48	10	0·04
Mouth	138	0·59	37	0·14
Pharynx	122	0·53	35	0·14
Jaw	178	0·76	86	0·33
Liver (specified as primary)	19	0·08	22	0·08
,, (not so specified)	1,236	5·30	2272	8·76
Bile passages	243	1·04	984	3·80
Larynx	430	1·84	77	0·30
Lung	1,291	5·54	369	1·42
Prostate	1,336	5·73	—	—
Penis	104	0·45	—	—
Bladder	594	2·55	278	1·07
Testis	119	0·51	—	—
Kidney	114	0·49	68	0·26
Skin	337	1·45	309	1·19
Pancreas	271	1·16	259	1·00
Breast	37	0·16	4146	15·98
Cervix uteri	—	—	1272	4·91
Corpus uteri	—	—	737	2·84
Uterus (undefined)	—	—	504	1·94
Ovary	—	—	1026	3·96
Vagina and vulva	—	—	258	1·00
Group 53	871	3·73	929	3·59
All sites	23,318	100·00	25,927	100·00

(Group 53 consists of carcinomas of sites which are only rarely affected).

The percentage distribution according to organ affected in men forms the basis for calculation of the expected deaths in Table XII.

Table II shows approximately the relative frequency of carcinoma of various sites with increasing age—approximately, for the percentage figures have this fault, that if one frequency is falling, the other percentages automatically rise; no value therefore should be attached to small differences. Only those organs which are affected with significant frequency are included in the table.

Ratio of Males to Females.

The comparative frequencies for the most important organs are shown in Table III.

In general one might say that, taking no account of the genital organs, the "organs of entry and exit" are more commonly affected in men, and other organs in women. Environment and occupation may, therefore, be expected to exert a greater influence in men.

Average Age at Death from Carcinoma of Each Site.

For each organ we can assess not only the frequency with which it is attacked, but also the average age at which this occurs. We are accustomed to speak of an average age in the case of a phenomenon which occurs more frequently with increasing age, reaches a maximum, and decreases again with further advancing years to become practically *nil*. Carcinoma is not a phenomenon of this kind;

TABLE II.—*Age Distribution of Carcinoma in Various Organs of Men and Women.*

	Men.			
	40–49.	50–59.	60–69.	70 and over.
Stomach	42·3	45·3	48·0	44·6
Rectum	7·4	7·0	5·9	6·6
Oesophagus	3·9	5·3	7·0	7·6
Intestines	7·4	7·4	6·9	7·8
Liver	4·9	5·3	5·2	5·4
Larynx	1·3	2·3	1·8	1·4
Lung	11·3	8·9	5·9	2·8
Prostate	1·2	2·2	5·3	8·4
Bladder	1·5	2·0	2·0	3·3
Skin	0·7	0·9	1·0	2·1
Other sites	18·1	13·4	11·0	10·0

	Women.			
	40–49.	50–59.	60–69.	70 and over.
Stomach	14·5	20·9	30·8	38·0
Rectum	4·2	4·6	4·6	5·0
Oesophagus	1·1	1·5	2·0	3·1
Intestines	5·4	6·8	9·9	12·3
Gall-bladder	2·3	3·9	4·8	3·6
Liver	4·6	7·0	9·9	10·5
Lung	1·9	1·8	1·6	0·7
Breast	31·4	22·9	13·7	8·3
Ovary	7·9	5·9	3·4	1·6
Cervix uteri	11·1	7·2	3·5	2·1
Corpus uteri	3·7	4·0	3·0	1·6
Other sites	11·9	13·5	15·8	13·2

TABLE III.—*Comparative Frequencies.*

	Male.	Female.
Stomach	1·40	1
Rectum	1·24	1
Oesophagus	2·75	1
Intestines	0·70	1
Peritoneum	0·39	1
Tongue	4·04	1
Lip	11·10	1
Mouth	3·73	1
Pharynx	3·49	1
Liver	0·53	1
Bile passages	0·25	1
Larynx	5·58	1
Lung	3·50	1
Bladder	2·14	1
Skin	1·09	1
Pancreas	1·05	1

a rise does indeed occur with increasing age, but the incidence continues to increase and the subsequent fall does not occur. Yet we can still speak of an average age, computed as follows :

> Suppose we find in the age-class 30–39 a cases of carcinoma ; in the age-class 40–49, b cases ; in the class 50–59, c ; in the class 60–69, d ; and in the class 70 and over, e cases of carcinoma. Then the average age of these cases of carcinoma will be approximately :

$$\frac{(a \times 35) + (b \times 45) + (c \times 55) + (d \times 65) + (e \times 80)}{a + b + c + d + e}$$

The average ages at death for carcinomas in various organs of men and women computed in this way are shown in Table IV.

TABLE IV.—*Average Age at Death.*

	Men.	Women.
Cervix uteri	—	56·1
Ovary	—	57·2
Breast	—	58·6
Lung	61·8	60·3
Corpus uteri	—	60·7
Pancreas	62·7	61·4
Rectum	66·4	65·1
Bile passages	69·7	66·2
Intestines	67·6	67·9
Stomach	67·8	68·4
Liver	68·2	68·0
Oesophagus	70·0	69·7
Bladder	71·0	69·8
Prostate	71·8	—
Skin	72·1	72·6
Penis	72·4	—

From this it appears that :

(1) In women carcinomas of the reproductive organs have the youngest average age, but in men they have the highest. (The average age of tumours of the testis cannot be calculated, because on the mortality cards they are not clearly divided into carcinomas, mixed tumours, seminomas, etc.).

(2) The average for other organs in men and women is about the same, except for the bile passages ; and the order of the organs, arranged according to their average and excepting the bile passages, is the same.

Death Rates by Age-classes for the Different Organs.

For each site we have calculated the number of deaths per year per 100,000 living people belonging to each age-class. The results are shown in Table V.

From this table it appears that :

(1) The total death-rates in the three lowest age-groups are higher for women than for men ; but in the two highest age-groups they are higher for men than for women.

(2) Although in general the frequency of carcinoma increases as age advances, in some sites the frequency decreases with advancing years (ovary and lung in

TABLE V.—*Deaths per Year per* 100,000 *Living in each Age-class.*

	Men.				
	30–39.	40–49.	50–59.	60–69.	70 and over.
All sites	15·57	61·64	219·54	640·08	1363·19
Stomach	5·66	27·19	99·71	308·01	608·33
Oesophagus	—	2·47	11·61	45·14	104·16
Rectum	1·76	4·71	15·58	37·97	87·50
Intestines	1·59	4·71	16·14	44·30	106·93
Liver	—	3·14	11·89	33·75	74·60
Lung	1·23	7·19	19·59	37·97	38·88
Larynx	—	—	5·38	11·39	20·13
Prostate	—	—	5·09	34·17	114·58
Bladder	—	0·89	4·53	13·50	45·83
Skin	—	—	1·98	6·32	29·16
	Women.				
	30–39.	40–49.	50–59.	60–69.	70 and over.
All sites	28·74	113·60	294·52	610·40	1154·03
Breast	7·99	35·79	67·39	84·00	96·89
Ovary	1·87	9·11	17·53	21·25	18·63
Cervix uteri	3·91	12·79	21·09	21·60	24·22
Corpus uteri	—	4·34	12·05	18·80	19·31
Stomach	4·93	16·48	61·91	188·00	439·13
Rectum	1·70	4·77	13·69	28·40	38·38
Oesophagus	—	1·30	4·38	12·40	36·64
Intestine	1·87	6·29	20·23	60·40	142·85
Liver	—	5·42	20·82	60·40	122·35
Gall-bladder	—	3·81	11·50	29·60	42·23
Lung	—	2·17	5·20	10·00	8·69

women). In some sites (lung in men, corpus uteri, cervix and breast) the increase of frequency in the highest age-groups becomes small, while in others (prostate, intestine, oesophagus, bladder and skin) the increment of frequency is always rising.

If it were only the ovary, breast and corpus uteri which showed a diminishing rate of increase, one might think of a functional decline of those organs, but at 60 the lungs are surely not functionally worn out. It may be that the carcinoma frequency of every organ has its rise, culminating point and fall; but for some organs (prostate, intestines, etc.) people do not live long enough in sufficient numbers to demonstrate this decrease in frequency. A decrease in frequency of carcinoma at a higher age, as some investigators suggest, need not be ascribed to wrong diagnosis or less profound medical interest in the older individual.

TABLE VI.—*Percentage Unmarried.*

	Men.				
	40–49.	50–59.	60–69.	70 and over.	All ages.
Intestines	—	9·7	9·5	8·3	—
Liver	—	10·0	11·1	6·5	—
Bile passages	—	—	—	—	9·9
Pancreas	—	—	—	—	8·5
Skin	—	—	—	—	17·5
Penis	—	—	—	—	7·6
Prostate	—	—	7·6	5·4	—
Bladder	—	—	8·6	6·9	—
Lung	10·1	9·5	5·9	7·7	—
Oesophagus	—	11·7	12·3	8·2	—
Rectum	11·4	9·4	7·9	8·9	—
Stomach	13·2	10·7	10·1	8·5	—
Larynx	—	12·8	12·1	5·6	—

	Women.				
	40–49.	50–59.	60–69.	70 and over.	All ages.
Intestines	—	13·8	15·1	13·2	—
Bile passages	—	5·2	3·6	7·6	—
Breast	16·8	19·9	20·6	21·6	—
Cervix	3·0	5·9	10·7	9·2	—
Corpus uteri	—	13·2	16·1	21·0	—
Ovary	20·5	21·0	17·3	20·6	—
Stomach	16·7	14·9	11·8	12·8	—
Lung	—	21·6	23·8	12·5	—
Rectum	—	13·6	14·9	12·1	—
Vagina and vulva	—	—	—	—	9·7
Liver	17·0	13·6	9·9	9·5	—
Bladder	—	—	—	—	15·5
Skin	—	—	—	—	14·8
Pancreas	—	—	—	—	11·9
Oesophagus	—	—	—	—	10·7

Influence of married state.

The percentages of unmarried people in the average populations (according to the 1930 census) were :

	40–49.	50–59.	60–69.	70 and over.
Men	11·2	10·3	10·2	8·8
Women	15·6	14·2	13·8	12·5

Table VI shows for every age-class the percentage unmarried of the people who died from carcinoma of the various organs.

Exercising the necessary caution, we may infer from Table VI that—

(1) Carcinomas of the prostate and penis are found more frequently in married people (including those that have been married), and carcinoma of the skin in unmarried men.

(2) Carcinomas of the breast, body of uterus, ovary and lung occur especially among unmarried women, as also of the bladder and skin in a lesser degree, whereas cancer of the vagina, cervix and gall-bladder are distinctly commoner in married women.

TABLE VII.—*Incidence of Carcinoma in the Various Organs of Married and Single Women.*

	Married.		Single.	
	Number.	Percentage of total.	Number.	Percentage of total.
Oesophagus	234	1·81	57	1·68
Stomach	3,466	26·77	917	27·09
Intestines	1,113	8·60	340	10·05
Rectum	576	4·45	158	4·70
Larynx	40	0·31	11	0·33
Lung	211	1·63	69	2·03
Tongue	28	0·22	9	0·27
Mouth	51	0·39	5	0·15
Jaw	48	0·37	11	0·33
Pharynx	20	0·15	6	0·18
Liver	1,123	8·67	242	7·15
Bile passages	563	4·34	56	1·65
Pancreas	158	1·22	28	0·83
Peritoneum	182	1·41	46	1·36
Kidney	32	0·25	9	0·27
Bladder	123	0·95	43	1·27
Corpus uteri	533	4·12	112	3·31
Cervix uteri	766	5·92	79	2·33
Uterus n.o.s.	247	1·91	79	2·33
Vagina and vulva	124	0·96	24	0·71
Skin	98	0·76	38	1·12
Breast	2,209	17·06	740	21·87
Ovary	550	4·25	188	5·56
Other sites	453	3·48	117	3·45
All sites	12,948	100·00	3384	100·00

(3) Carcinomas of the rectum, stomach and intestines show little association with wedlock.

We may note that the average age of mammary carcinoma is 58·6. Although after 45 there is practically no difference in the circumstances of the breast in married and unmarried women, yet in the higher age-groups the difference in frequency between the married and unmarried persists or is even greater. In my opinion this points to the existence of a long latent period, which we know exists in certain occupational cancers. The same holds good for carcinoma of the cervix, body of uterus and ovary.

The percentage distributions according to the organ affected in married and single women are shown in Table VII, and these form the bases for calculation of the expected deaths in Tables XIII*a* and XIII*b* respectively.

Carcinoma in towns.

Taking into account the age-group structure and the ratio between married and unmarried people in the towns, we can calculate the total mortality which might be expected if the mortality in towns were equal to that of the average population for the different sites. We should expect 12,875 deaths, whereas in reality we find 14,193 deaths, that is, an excess of 10·3 per cent. If we compute the mortality of the different organs in this way, it appears that in the big towns (which had more than 100,000 inhabitants on January 1, 1931) stomach and skin carcinoma mortalities are considerably lower, but for the tongue, larynx, lung, uterus, ovary and breast they are considerably higher.

The percentage distributions of the organs affected in the big towns and in the country, for men and women together, are shown in Table VIII.

TABLE VIII.—*Percentage Distributions in Big Towns and in Country.*

	Town.	Country.
Mouth cavity	1·8	1·5
Tongue	0·8	0·4
Oesophagus	4·2	4·1
Stomach	29·8	38·4
Rectum	5·8	5·1
Liver, gall-bladder, etc.	7·9	10·2
Pancreas	1·2	0·9
Peritoneum	1·1	0·8
Intestines	9·2	8·0
Larynx	1·3	0·8
Lung and mediastinum	4·7	2·9
Uterus	7·0	4·3
Ovary	2·5	1·9
Breast	9·7	7·7
Male urogenital	5·3	4·5
Skin	0·8	1·6
Other sites	6·9	6·9

Jews and carcinoma.

The distribution of deaths over the different organs in Jews is shown in Table IX.

We find a low figure for gastric carcinoma among Jews and high figures for carcinomas of the intestines, rectum, lung and bladder. Among Jewesses we find a low incidence for carcinomas of the stomach, bile passages and cervix, and a high incidence for carcinomas of the breast, ovary, intestines and kidneys.

It would be more correct to compare the Jews, not with the average population, but with that of the big towns, for 80·4 per cent and 80·7 per cent of Jews and Jewesses respectively live in the big towns. If we do so, we find an increased incidence for carcinomas of the liver, gall-bladder, intestines, rectum, lung, ovary and breast, and a decrease for the mouth, oesophagus, stomach, uterus and skin.

From the age-group distribution of Jews and Jewesses we should expect 228 and 296 cases of carcinoma respectively. We find, however, 246 and 368, that is to say 7·9 per cent and 24·3 per cent more than in the average population. So we must conclude that whilst Jews yield about as many cases of carcinoma as their environment leads us to expect, Jewesses on the other hand yield considerably more.

TABLE IX.—*Distribution of Deaths over Different Organs in Jews.*

	Men.		Women.	
	Actual.	Expected.	Actual.	Expected.
Oesophagus	15	16·4	6	8·0
Stomach	64	111·6	70	107·3
Intestines	33	18·4	53	35·4
Rectum	26	16·2	18	17·6
Larynx	4	4·5	1	1·1
Lung	30	13·6	7	5·2
Lip	1	1·2	—	0·1
Mouth	2	1·5	—	0·5
Tongue	4	2·3	—	0·8
Jaw	3	1·9	1	1·2
Pharynx	1	1·3	—	0·5
Liver	13	13·2	29	33·3
Bile-passages	5	2·6	7	14·0
Pancreas	4	2·9	2	3·7
Peritoneum	1	1·4	4	4·7
Kidney	3	1·2	5	0·3
Bladder	12	6·3	7	3·9
Prostate	15	14·1	—	—
Penis	—	1·1	—	—
Corpus uteri	—	—	17	10·5
Cervix uteri	—	—	9	18·1
Uterus (undefined)	—	—	6	7·1
Vagina and vulva	—	—	4	3·7
Breast	—	—	81	58·8
Ovary	—	—	28	14·6
Skin	1	3·6	1	4·4
Other sites	9	10·9	12	13·2
	246	246	368	368

Carcinoma and trade or profession.

We now come to the real object of this research—to trace the influence of trade or profession on the origin and the localization of carcinoma. Our research covers a wider field than that of the so-called occupational cancers, and all that has gone before is necessary to enable us to interpret what follows.

Before we can proceed, we shall have to examine some problems a little more closely. This will be facilitated by taking a specific occupation as an example, and I have chosen coal-mining.

The mortality cards show us—

A. How many miners died of carcinoma from 1931 to 1935, their ages, and the sites of their carcinomas.

B. How many people who had formerly been miners died of carcinoma from 1931 to 1935, their ages, and the sites of their carcinomas.

The census of 1930 (there was no census in 1940 on account of the German occupation, consequently an interpolation is impossible for the years between 1930 and 1940) shows us—

How many miners there were in the Netherlands and their ages, but nothing about ex-miners.

Now if we take groups A and B together we include all miners who got carcinoma while still working or after leaving the trade, with their ages, but their distribution over the various age-classes cannot be compared with the census of 1930, which gives no information about ex-miners. We can best get over this difficulty by expressing the actual deaths for each site as percentages of those for all sites, and comparing the distribution for each occupation with that for all males ; the drawbacks to this plan are that the percentages are mutually dependent, and if the percentage of one side rises, the other percentages automatically fall, and also that we cannot form an opinion about the absolute frequency of carcinoma in the different trades.

On the other hand, if we only take group A there are the difficulties that the number of cases of carcinoma for each trade will be smaller, and that miners, especially the older ones, leave off working when they fall ill with carcinoma, and consequently on the mortality cards they are stated as " occupation—none." The latter difficulty might be overcome by considering only those miners who died under the age of 60 (before this age they cannot give up their trade for financial reasons) ; but then the numbers available become much smaller, and those carcinomas whose average age lies over 60 will be left out of account.

In my opinion the best way is to use both methods, the latter with the correction that only the cases in the age-groups 30–39, 40–49 and 50–59 are taken into account. In this way we get a comparison of percentages of the greatest possible number of cases, and absolute figures besides.

The percentage distribution of carcinomas over the various organs in coal-miners, both in employment and formerly employed, is shown in Table X.

The column " to be expected " in Table X shows how the distribution of the cases of carcinoma in a certain grade over the different organs would be, if the distribution we found for the whole male (or female) population applied.

TABLE X.—*Percentage Distribution of Carcinomas over the Organs in Coal-miners.*

	In employment.	Formerly employed.	Total.	To be expected.
Stomach	39	29	68	45·4
Rectum	2	1	3	6·6
Oesophagus	3	1	4	6·7
Lung	5	3	8	5·5
Intestines	1	2	3	7·5
Liver	2	4	6	5·4
Bile-passages	—	2	2	1·0
Larynx	—	1	1	1·8
Peritoneum	—	1	1	0·6
Prostate	—	1	1	5·7
Bladder	—	1	1	2·5
Other sites	1	1	2	11·3
	53	47	100	100

To decide whether the differences found are real or not the average error must be calculated. This has been done following the method of Dr. Bruno Scholz (*Methodik der medizinischen Erforschung*, page 133). Suppose we find a total of x cases of carcinoma in a certain trade. Of these, y cases are carcinomas of the stomach. Then the average error is $\sqrt{\frac{y \times (x-y)}{x}}$. When y is small this is practically equal to $\sqrt{y}$.

This average error has always been taken into account in this research, and a difference is called real only when the percentages found and those to be expected differ by at least 3 times the average error.

We find for miners a high frequency of carcinoma of the stomach and a low prostate frequency. In the age-classes 30–39, 40–49 and 50–59 we find a total mortality of 50 cases, whereas we should expect 32, so that the total carcinoma mortality of coal miners is very high. The frequency of carcinoma of the stomach is greatly increased, for we find 33 cases instead of the 12 we should expect. The number of cases of carcinoma of the prostate in these age-classes is too small to reach a conclusion.

The question now arises, is this high stomach frequency amongst miners bound up with their trade or with their environment; is it an "occupational cancer" or a "social cancer"? (Cramer). To answer this question we must study "wives of miners" (from the mortality cards of those married women where the trade of the head of the family is given as "coal miner." The married woman practically always lodges with her husband).

The percentage distribution of carcinoma over the various organs in "wives of miners" compared with the distribution in married women amongst the general population is shown in Table XI.

Taking the "average error" into account, we see from Table XI that only

carcinoma of the cervix is significantly commoner in miners' wives; carcinoma of the stomach is not. Women, as a rule, do not share the occupational consequences with their husbands, but only the domestic circumstances (house, standard of living, etc.), so that the higher stomach frequency amongst miners is a result of their occupation in the proper sense.

TABLE XI.—*Percentage Distribution in Wives of Miners.*

	Actual deaths.	Expected deaths.
Stomach	15	17
Rectum	3	3
Oesophagus	1	1
Lung	1	1
Intestines	3	6
Liver	3	6
Pancreas	2	1
Peritoneum	4	1
Cervix uteri	11	4
Corpus uteri	2	3
Uterus, undefined	4	1
Ovary	2	3
Vagina and vulva	1	1
Breast	11	11
Other sites	2	6

Before giving the tables showing the number of cases of carcinoma for the different trades I would like to emphasize the importance of keeping the trades separate, and not grouping them. The smaller the unit in our research the more chance we have of getting results of value. If we gather the trades into groups their typical characteristics will become less and less obvious until at last the ratios obtaining among the average population are reached. I have tried taking some trades together, but the typical characteristics of a certain trade always get lost, and the whole group misses what individual component parts show as typical.

It is, however, worth while comparing any one trade with another of the same kind, and I have therefore tried to arrange the order of the trades discussed so that trades of a kind come near together.

Table XII compares the actual deaths of men of different occupations with the numbers expected if the total carcinoma deaths had in each instance been distributed over the various sites in the same way as amongst all males—that is to say, according to the percentages shown in Table I.

Table XIII*a* gives a similar comparison, for wives of men in different occupations, with the deaths expected if the total carcinoma deaths had been distributed in the same way as amongst all married women. Table XIII*b* compares the deaths in a few occupations of single women with those expected from the site distributions amongst all single women.

TABLE XII.—*Recorded and Expected Deaths from Carcinoma amongst Men of Various Trades and Professions.*

Site of carcinoma.	*Publicans, Hotel keepers, Waiters, Coffeehouse keepers.*		*Head-teachers and Teachers.*		*Butchers and Butchers' men.*		*Carpenters.*		*Bakers, Confectioners and their Assistants.*		*Smiths (Farrier Stovemakers and their Assistants).*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	58	26·5	11	14·7	15	16·4	53	44·7	19	25·0	10	14·7
Stomach	119	180·1	78	99·8	93	111·6	312	304·0	172	170·1	96	100·3
Intestines	22	29·7	32	16·5	23	18·4	48	50·2	38	28·1	19	16·6
Rectum	22	26·1	15	14·5	17	16·2	52	44·0	30	24·6	17	14·5
Larynx	21	7·3	4	4·0	4	4·5	14	12·3	8	6·9	5	4·1
Lung	33	22·0	12	12·2	26	13·6	35	37·1	20	20·8	8	12·2
Lip	—	1·9	—	1·1	2	1·1	—	3·2	—	1·8	—	1·1
Tongue	10	4·0	3	2·1	3	2·3	3	6·4	4	3·6	2	2·1
Mouth	7	2·3	3	1·3	—	1·5	4	4·0	—	2·2	—	1·3
Jaw	4	3·0	2	1·7	4	1·9	5	5·1	4	2·9	6	1·7
Pharynx	8	2·1	2	1·2	2	1·3	4	3·6	—	2·0	2	1·2
Liver	19	21·4	22	11·8	5	13·2	34	36·0	19	20·2	9	11·9
Bile passages	3	4·1	3	2·3	3	2·5	8	7·0	6	3·9	2	2·3
Pancreas	6	4·6	5	2·6	5	2·9	6	7·8	3	4·4	6	2·6
Peritoneum	3	2·2	—	1·2	1	1·4	2	3·8	1	2·1	1	1·2
Kidney	2	1·9	1	1·1	1	1·2	—	3·3	—	1·8	—	1·1
Bladder	13	10·1	5	5·6	10	6·3	16	17·1	11	9·6	8	5·6
Penis	2	1·8	1	1·0	1	1·1	7	3·0	1	1·8	7	1·0
Prostate	23	22·7	13	12·6	16	14·1	39	38·4	25	21·5	16	12·7
Skin	3	5·8	1	3·2	2	3·6	12	9·7	4	5·4	1	3·2
Other sites	19	17·4	7	9·5	13	10·9	16	29·1	10	16·3	6	9·6
Total	397	397	220	220	246	246	670	670	375	375	221	221

Site of carcinoma.	*Shoemakers and Shoe hands.*		*Soldiers (Land and Naval Forces).*		*Farmers.*		*Agricultural Labourers.*		*Stock farmers.*		*Stock farmers' hands.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	21	21·1	19	18·5	171	186.8	106	125·1	36	36·0	2	3·9
Stomach	138	143·8	86	125·7	1566	1270·8	1092	850·8	314	244·5	33	26·3
Intestines	26	23·7	27	20·7	163	209·8	125	140·4	25	40·4	3	4·3
Rectum	26	20·8	18	18·2	166	184·0	81	123·2	26	35·4	3	3·8
Larynx	8	5·8	18	5·1	12	51·5	11	34·5	7	9·9	—	1·1
Lung	10	17·6	15	15·3	63	155·2	38	103·9	21	29·9	4	3·2
Lip	—	1·5	1	1·3	25	13·4	12	9·0	3	2·6	—	0·3
Tongue	2	3·0	6	2·6	11	26·6	6	17·8	1	5·1	—	0·6
Mouth	5	1·9	3	1·6	10	16·5	7	11·1	2	3·2	—	0·3
Jaw	1	2·4	3	2·1	17	21·3	9	14·2	1	4·1	—	0·4
Pharynx	1	1·7	3	1·5	10	14·8	4	9·9	—	2·9	—	0·3
Liver	20	17·1	24	14·9	159	150·7	97	100·9	32	29·0	3	3·1
Bile passages	5	3·3	3	2·9	32	29·1	17	19·5	4	5·6	—	0·6
Pancreas	7	3·7	5	3·2	17	32·5	11	21·8	6	6·3	1	0·7
Peritoneum	—	1·8	—	1·6	8	15·7	7	11·1	4	3·0	—	0·3
Kidney	1	1·6	1	1·4	10	13·7	3	9·2	2	2·6	—	0·3
Bladder	14	8·1	11	7·1	56	71·4	45	47·8	2	13·7	2	1·5
Penis	2	1·4	2	1·2	10	12·6	3	8·4	—	2·4	—	0·3
Prostate	20	18·2	24	15·9	142	160·5	96	107·4	27	30·8	4	3·3
Skin	1	4·6	1	4·0	69	40·6	43	27·2	10	7·8	2	0·8
Other sites	9	13·9	7	12·2	64	123·4	62	82·0	16	23·8	1	2·6
Total	317	317	277	277	2801	2801	1875	1875	539	539	58	58

Note.—A. = Number of deaths actually recorded.
E. = Number of deaths expected from the site distribution of carcinoma deaths amongst all males (Table I).

TABLE XII—(*cont.*).

Site of carcinoma.	*House Painters and their hands.*		*Weavers and Workers in woollens.*		*University men (Physicians, Clergymen, Engineers, Lawyers).*		*Roman Catholic Priests.*		*General Labourers.*		*Masters (Secondary and Grammar schools).*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	24	20·2	9	14·1	6	11·1	3	5·2	88	63·8	3	4·7
Stomach	139	137·5	116	96·2	36	75·3	39	35·4	448	433·7	15	32·2
Intestines	18	22·7	11	15·9	18	12·4	6	5·8	57	71·6	11	5·3
Rectum	19	19·8	12	14·0	12	10·9	6	5·1	58	62·8	6	4·7
Larynx	8	5·6	4	3·9	6	3·1	2	1·4	21	17·6	1	1·3
Lung	17	16·8	5	11·7	25	9·2	3	4·3	27	53·0	10	3·9
Lip	1	1·4	—	1·0	—	0·8	—	0·4	6	4·6	—	0·3
Tongue	3	2·9	—	2·0	5	1·6	1	0·7	8	9·1	—	0·7
Mouth	—	1·8	1	1·3	1	1·0	—	0·5	8	5·6	1	0·4
Jaw	4	2·3	3	1·6	—	1·0	2	0·6	8	7·3	—	0·5
Pharynx	1	1·6	—	1·1	4	0·9	—	0·4	4	5·1	1	0·4
Liver	17	16·3	21	11·4	10	8·9	4	4·2	49	51·4	3	3·8
Bile passages	4	3·2	3	2·2	2	1·7	—	0·8	13	10·0	—	0·7
Pancreas	4	3·5	1	2·4	2	1·9	1	0·8	11	11·1	1	0·8
Peritoneum	1	1·7	—	1·2	1	0·9	1	0·4	5	5·4	—	0·4
Kidney	2	1·5	—	1·0	3	0·8	1	0·4	4	4·7	—	0·3
Bladder	4	7·7	5	5·4	5	4·2	2	2·0	21	24·4	3	1·8
Penis	3	1·4	2	0·9	—	0·7	—	0·4	8	4·3	3	0·3
Prostate	20	17·4	10	12·1	23	9·5	6	4·5	57	54·8	11	4·1
Skin	8	4·4	4	3·1	—	2·4	—	1·1	14	13·9	—	1·0
Other sites	6	13·3	5	9·5	7	7·3	1	3·6	41	41·8	2	3·4
Total	303	303	212	212	166	166	78	78	956	956	71	71

Site of carcinoma.	*Office staffs.*		*Tailors and Tailors' journeymen.*		*Drapers and Drapers' assistants.*		*Masons and their hands.*		*Dealers in fuel.*		*Bargemen.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	30	36·9	24	18·1	7	8·7	24	22·8	11	5·0	29	22·9
Stomach	170	250·9	113	122·9	41	59·5	160	155·2	35	34·0	171	156·1
Intestines	61	41·4	25	20·3	9	9·8	23	25·6	5	5·6	20	25·8
Rectum	45	36·3	18	17·8	15	8·6	18	22·5	—	4·9	16	22·6
Larynx	15	10·2	4	5·0	6	2·4	4	6·3	2	1·4	7	6·3
Lung	63	30·6	10	15·0	22	7·3	17	18·9	4	4·2	14	19·1
Lip	1	2·7	—	1·3	1	0·6	1	1·6	—	0·4	3	1·7
Tongue	9	5·3	3	2·6	1	1·2	6	3·2	—	0·7	5	3·3
Mouth	7	3·3	1	1·6	1	0·6	2	2·0	—	0·4	2	2·0
Jaw	4	4·2	1	2·1	2	0·8	3	2·6	—	0·6	1	2·6
Pharynx	8	2·9	—	1·4	—	1·0	2	1·8	1	0·4	1	1·8
Liver	39	29·8	17	14·6	8	7·1	19	18·4	2	4·0	15	18·5
Bile passages	2	5·7	4	2·8	3	1·4	3	3·6	1	0·8	3	3·6
Pancreas	10	6·4	1	3·2	4	1·5	2	4·0	2	0·9	5	4·0
Peritoneum	3	3·1	—	1·5	—	0·7	2	1·9	1	0·4	1	1·9
Kidney	6	2·7	3	1·3	3	0·6	—	1·7	—	0·4	2	1·7
Bladder	14	14·1	6	6·9	2	3·3	13	8·7	4	1·9	7	8·8
Penis	2	2·5	4	1·2	—	0·6	1	1·5	—	0·3	2	1·5
Prostate	42	31·7	28	15·5	5	7·5	27	19·6	4	4·3	13	19·7
Skin	3	8·0	1	3·9	—	1·9	3	5·0	—	1·1	12	5·0
Other sites	19	24·3	8	12·0	1	5·9	12	15·1	3	3·3	15	15·1
Total	553	553	271	271	131	131	342	342	75	75	344	344

TABLE XII—(*cont.*).

Site of carcinoma.	*Police.*		*Diamond cutters.*		*Carmen (Drivers, Stable keepers, Coachmen).*		*Chauffeurs.*		*Firemen and Engine drivers.*		*Dock labourers.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	12	8·5	7	7·7	16	11·5	1	2·2	16	16·3	8	6·3
Stomach	50	57·8	44	52·2	84	78·5	17	15·0	120	111·2	45	43·1
Intestines	5	9·5	8	8·6	4	13·0	3	2·5	18	18·4	7	7·1
Rectum	16	8·3	3	7·5	14	11·4	3	2·2	13	16·1	6	6·2
Larynx	3	2·3	2	2·1	5	3·2	—	0·6	8	4·5	3	1·7
Lung	10	7·0	15	6·4	4	9·6	4	1·8	12	13·6	1	5·3
Lip	3	0·6	—	0·5	2	0·8	—	0·2	—	1·2	1	0·5
Tongue	1	1·2	3	1·1	—	1·6	—	0·3	3	2·3	3	0·9
Mouth	—	0·7	1	0·7	2	1·0	1	0·2	1	1·4	1	0·6
Jaw	1	1·0	—	0·9	—	1·3	—	0·2	—	1·9	—	0·7
Pharynx	—	0·7	2	0·6	3	0·9	—	0·2	—	1·3	—	0·5
Liver	5	6·8	9	6·2	12	9·3	—	1·8	12	13·2	3	5·1
Bile passages	1	1·3	2	1·2	2	1·8	—	0·3	2	2·5	1	1·0
Pancreas	1	1·5	—	1·3	3	2·0	—	0·3	6	2·8	—	1·1
Peritoneum	—	0·7	1	0·6	1	1·0	2	0·2	—	1·4	1	0·5
Kidney	—	0·6	2	0·6	—	0·8	—	0·2	1	1·2	—	0·5
Bladder	2	3·2	4	2·9	11	4·4	—	0·8	10	6·2	1	2·4
Penis	2	0·6	—	0·5	1	0·8	—	0·1	—	1·1	2	0·4
Prostate	8	7·2	8	6·6	8	9·9	—	1·9	14	14·0	8	5·4
Skin	1	1·8	—	1·7	—	2·5	—	0·5	2	3·6	—	1·4
Other sites	6	5·7	4	5·1	1	7·7	2	1·5	7	10·8	4	4·3
Total	127	127	115	115	173	173	33	33	245	245	95	95

Site of carcinoma.	*Fishermen.*		*Green-grocers.*		*Commercial travellers.*		*Market gardeners and their assistants.*		*Navvies.*		*Hall porters and Caretakers.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	18	11·5	8	8·8	16	7·7	24	18·1	10	10·6	8	6·4
Stomach	76	78·0	74	59·9	39	52·6	140	123·4	95	72·1	33	43·6
Intestines	8	12·9	5	9·9	9	8·7	15	20·4	7	11·9	8	7·2
Rectum	6	11·3	6	8·7	7	7·6	19	17·9	10	10·4	10	6·3
Larynx	3	3·2	1	2·4	3	2·1	6	5·0	2	2·9	4	1·8
Lung	7	9·5	7	7·3	15	6·4	7	15·1	2	8·8	14	5·3
Lip	1	0·8	—	0·6	—	0·6	3	1·3	2	0·8	1	0·5
Tongue	2	1·6	—	1·3	1	1·1	1	2·6	—	1·5	2	0·9
Mouth	2	1·0	—	0·8	4	0·7	—	1·6	—	0·9	2	0·6
Jaw	4	1·2	—	1·0	—	0·9	—	2·1	1	1·2	—	0·7
Pharynx	—	0·9	2	0·7	1	0·6	1	1·4	—	0·8	—	0·5
Liver	11	9·3	10	7·1	5	6·1	12	14·6	12	8·6	3	5·1
Bile passages	1	1·8	—	1·4	—	1·2	4	2·8	1	1·7	1	1·0
Pancreas	2	2·0	2	1·5	4	1·3	3	3·2	2	1·8	—	1·1
Peritoneum	3	1·0	—	0·7	2	0·7	1	1·5	—	0·9	1	0·5
Kidney	2	0·8	2	0·6	2	0·6	—	1·3	1	0·8	—	0·5
Bladder	3	4·4	3	3·4	1	3·0	8	6·9	2	4·1	2	2·5
Penis	1	0·8	—	0·6	—	0·5	—	1·2	—	0·7	—	0·4
Prostate	13	9·9	6	7·6	3	6·6	19	15·6	3	9·1	5	5·5
Skin	5	2·5	3	1·9	—	1·7	5	4·0	2	2·3	—	1·4
Other sites	4	7·6	3	5·8	4	5·3	4	12·0	7	7·1	2	4·2
Total	172	172	132	132	116	116	272	272	159	159	96	96

TABLE XII—(*cont.*).

Site of carcinoma.	*Railway and Tram men.*		*Cigar makers and Cigar manufacturers.*		*Tobacconists.*		*Plumbers.*		*Printers.*		*Grocers.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	29	30·9	8	11·0	3	3·7	4	3·2	5	6·7	12	13·8
Stomach	211	210·5	74	74·9	26	25·4	14	21·8	34	45·4	86	93·9
Intestines	31	34·8	12	12·4	2	4·2	2	3·6	12	7·5	14	15·5
Rectum	34	30·5	7	10·8	5	3·7	4	3·2	10	6·6	12	13·6
Larynx	6	8·5	2	3·0	1	1·0	2	0·9	2	1·8	6	3·8
Lung	29	25·7	14	9·1	10	3·1	2	2·7	3	5·5	19	11·5
Lip	6	2·2	1	0·8	—	0·3	—	0·2	—	0·5	2	1·0
Tongue	7	4·4	1	1·6	3	0·5	1	0·5	2	1·0	1	2·0
Mouth	2	2·7	1	1·0	—	0·3	—	0·3	—	0·6	—	1·2
Jaw	8	3·5	—	1·2	1	0·4	—	0·4	—	0·8	3	1·6
Pharynx	3	2·5	1	0·9	1	0·3	—	0·3	—	0·5	1	1·1
Liver	23	25·0	10	8·9	1	3·0	5	2·6	7	5·4	13	11·1
Bile passages	4	4·8	1	1·7	—	0·6	—	0·5	3	1·0	5	2·2
Pancreas	7	5·4	2	1·9	1	0·6	3	0·5	2	1·2	2	2·4
Peritoneum	3	2·6	1	0·9	—	0·3	—	0·3	—	0·6	—	1·2
Kidney	2	2·3	2	0·8	—	0·3	2	0·2	2	0·5	—	1·0
Bladder	14	11·8	5	4·2	—	1·4	3	1·2	5	2·5	9	5·3
Penis	3	2·1	1	0·7	—	0·3	1	0·2	—	0·4	1	0·9
Prostate	24	26·6	12	9·4	1	3·2	4	2·7	7	5·7	6	11·9
Skin	1	6·7	2	2·4	—	0·8	—	0·7	2	1·4	4	3·0
Other sites	17	20·5	8	7·4	1	2·6	1	2·0	4	4·4	11	9·0
Total	464	464	165	165	56	56	48	48	100	100	207	207

TABLE XIII*a*.—*Recorded and Expected Deaths from Carcinoma amongst Wives of Men in Various Trades and Professions.*

Site of carcinoma.	*Publicans' and Hotel keepers' wives.*		*Head-teachers' and Teachers' wives.*		*Butchers' wives.*		*Carpenters' wives.*		*Bakers' and Confectioners' wives.*		*Smiths' wives.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	—	2·4	—	1·1	1	1·4	4	4·9	2	2·6	1	1·2
Stomach	29	34·8	11	16·4	18	20·6	60	72·5	39	37·5	17	17·4
Intestines	10	11·2	5	5·2	7	6·6	21	23·3	13	12·0	6	5·6
Rectum	6	5·8	2	2·7	3	3·4	16	12·1	7	6·2	2	2·9
Larynx	1	0·4	—	0·2	—	0·2	—	0·8	1	0·4	1	0·2
Lung	1	2·1	—	1·0	2	1·2	2	4·4	2	2·3	4	1·1
Tongue	—	0·3	—	0·1	—	0·2	—	0·6	—	0·3	—	0·1
Mouth	—	0·5	—	0·2	—	0·3	1	1·0	—	0·5	—	0·3
Jaw	1	0·5	—	0·2	—	0·3	4	1·1	—	0·5	—	0·2
Pharynx	—	0·2	—	0·1	—	0·1	1	0·4	—	0·2	1	0·1
Liver	8	11·3	4	5·3	3	6·7	22	23·5	10	12·1	8	5·6
Bile passages	8	5·6	—	2·6	—	3·3	14	11·8	6	6·1	2	2·8
Pancreas	2	1·6	—	0·7	1	0·9	3	3·3	3	1·7	—	0·8
Peritoneum	—	1·8	1	0·9	1	1·1	3	3·8	4	2·0	—	0·9
Kidney	—	0·3	—	0·2	1	0·2	—	0·7	—	0·4	—	0·2
Bladder	—	1·2	—	0·6	—	0·7	1	2·6	—	1·3	1	0·6
Corpus uteri	8	5·4	3	2·5	3	3·2	10	11·1	2	5·8	1	2·7
Cervix uteri	14	7·7	3	3·6	11	4·6	19	16·0	7	8·3	5	3·8
Uterus n.o.s.	5	2·5	2	1·2	2	1·5	8	5·2	4	2·7	—	1·2
Vagina and vulva	1	1·2	—	0·6	1	0·7	4	2·6	1	1·3	2	0·6
Skin	1	1·0	—	0·5	1	0·6	1	2·1	2	1·1	—	0·5
Breast	34	22·2	24	10·4	15	13·1	53	46·2	25	23·9	12	11·1
Ovary	—	5·5	4	2·6	7	3·3	13	11·5	7	5·9	1	2·8
Other sites	1	4·6	2	2·2	—	2·8	9	9·5	5	4·9	1	2·3
Total	130	130	61	61	77	77	271	271	140	140	65	65

Note.—A. = Number of deaths actually recorded.
E. = Number of deaths expected from the site distribution of carcinoma deaths amongst all married women.

TABLE I—(*cont.*).

Site of carcinoma.	*Shoe makers' wives.*		*Farmers' wives.*		*Farm labourers' wives.*		*Stock farmers' wives.*		*House painters' wives.*		*Weavers' wives.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	1	2·8	27	21·4	16	7·7	6	3·9	2	2·4	2	1·3
Stomach	56	41·0	370	316·7	176	113·5	61	58·1	39	35·6	26	19·3
Intestines	9	13·2	107	101·7	22	36·5	25	18·7	10	11·4	10	6·2
Rectum	9	6·8	45	52·6	15	18·9	8	9·7	2	5·9	3	3·2
Larynx	—	0·5	5	3·7	—	1·3	1	0·7	—	0·4	—	0·2
Lung	3	2·5	14	19·3	2	6·9	3	3·5	1	2·2	2	1·2
Tongue	—	0·3	1	2·6	2	0·9	2	0·5	—	0·3	—	0·2
Mouth	—	0·6	1	4·6	—	1·7	—	0·8	—	0·5	—	0·3
Jaw	—	0·6	4	4·4	2	1·6	1	0·8	—	0·5	—	0·3
Pharynx	—	0·2	1	1·8	—	0·6	1	0·3	—	0·2	—	0·1
Liver	8	13·3	166	102.6	47	36·8	17	18·8	10	11·5	11	6·2
Bile passages	8	6·6	64	51·3	14	18·4	11	9·4	6	5·8	3	3·1
Pancreas	1	1·9	8	14·4	6	5·2	3	2·6	—	1·6	1	0·9
Peritoneum	5	2·2	10	16·7	1	6·0	4	3·1	2	1·9	1	1·1
Kidney	—	0·4	2	3·6	2	1·1	1	0·5	1	0·3	—	0·2
Bladder	1	1·5	3	11·2	3	4·0	1	2·1	—	1·3	—	0·7
Corpus uteri	5	6·3	46	48·7	8	17·5	6	8·9	2	5·5	2	3·0
Cervix uteri	5	9·1	20	70·0	8	25·1	4	12·8	14	7·9	2	4·3
Uterus n.o.s.	—	2·9	12	22·6	7	8·1	2	4·1	1	2·5	1	1·4
Vagina and vulva	1	1·5	5	11·4	—	4·1	1	2·1	3	1·3	1	0·7
Skin	2	1·2	10	9·0	3	3·2	—	1·6	—	1·0	—	0·5
Breast	26	26·1	181	201·8	67	72·3	41	37·0	33	22·7	4	12·2
Ovary	7	6·5	43	50·3	9	18·0	18	9·2	3	5·7	2	3·1
Other sites	6	5·0	44	41·2	14	14·6	—	7·8	4	4·6	2	2·3
Total	153	153	1183	1183	424	424	217	217	133	133	72	72

Site of carcinoma.	*Wives of University men.*		*General Labourers' wives.*		*Wives of Office staffs.*		*Tailors' wives.*		*Masons' wives.*		*Wives of Dealers in fuel.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	—	1·3	7	8·1	1	3·4	5	2·3	—	2·4	2	1·1
Stomach	7	18·7	121	120·2	20	54·4	31	34·3	40	35·1	14	16·1
Intestines	10	6·0	38	38·6	28	17·3	7	11·0	14	11·3	5	5·2
Rectum	4	3·1	22	20·0	7	8·9	6	5·7	11	5·8	2	2·7
Larynx	—	0·2	2	1·4	1	0·6	—	0·4	—	0·4	2	0·2
Lung	—	1·1	7	7·3	4	3·3	1	2·1	—	2·1	1	1·0
Tongue	—	0·2	—	1·0	—	0·4	1	0·3	—	0·3	—	0·1
Mouth	—	0·3	—	1·8	—	0·8	—	0·5	—	0·5	—	0·2
Jaw	—	0·3	1	1·7	—	0·7	1	0·5	—	0·5	1	0·2
Pharynx	—	0·1	—	0·7	—	0·3	—	0·2	—	0·2	—	0·1
Liver	1	6·1	37	38·9	10	17·4	8	11·1	6	11·4	—	5·2
Bile passages	2	3·0	23	19·5	4	8·7	8	5·6	6	5·7	6	2·6
Pancreas	—	0·9	3	5·5	4	2·5	1	1·6	—	1·6	1	0·7
Peritoneum	2	1·0	7	6·3	4	2·8	4	1·8	2	1·8	—	0·8
Kidney	—	0·2	—	1·1	2	0·5	—	0·3	—	0·3	—	0·1
Bladder	1	0·7	3	4·3	1	1·9	2	1·2	2	1·2	1	0·6
Corpus uteri	5	2·9	15	18·5	8	8·3	5	5·3	3	5·4	2	2·5
Cervix uteri	2	4·1	35	26·6	10	11·9	5	7·6	7	7·8	7	3·6
Uterus n.o.s.	2	1·3	12	8·6	5	3·8	5	2·4	3	2·5	3	1·1
Vagina and vulva	—	0·7	2	4·3	3	1·9	2	1·2	—	1·3	1	0·6
Skin	—	6·5	8	3·4	1	1·5	1	1·0	2	1·0	1	0·5
Breast	18	11·9	75	76·6	58	34·3	26	21·8	25	22·3	5	10·2
Ovary	11	3·0	18	19·0	27	8·5	7	5·4	6	5·6	3	2·5
Other sites	5	2·4	13	15·6	3	6·9	2	4·4	4	4·5	3	2·1
Total	70	70	449	449	201	201	128	128	131	131	60	60

TABLE XIII*a*—(*cont.*).

Site of carcinoma.	*Wives of Bargemen*		*Police wives.*		*Wives of Diamond cutters.*		*Carmen's wives.*		*Chauffeurs' wives.*		*Wives of firemen.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	4	2·0	—	0·7	—	0·9	1	0·7	1	0·7	—	1·9
Stomach	17	30·2	8	10·7	14	12·6	13	9·6	7	9·6	16	28·1
Intestines	9	9·7	3	3·4	4	4·0	3	3·1	3	3·1	8	9·0
Rectum	5	5·0	1	1·8	2	2·1	—	1·6	1	1·6	3	4·7
Larynx	—	0·4	—	0·1	—	0·1	—	0·1	—	0·1	2	0·3
Lung	3	1·8	—	0·7	1	0·8	1	0·6	1	0·6	1	1·7
Tongue	2	0·2	—	0·1	—	0·1	—	0·1	1	0·1	—	0·2
Mouth	1	0·4	—	0·2	—	0·2	—	0·1	—	0·1	—	0·4
Jaw	—	0·4	1	0·1	—	0·2	—	0·1	—	0·1	1	0·4
Pharynx	—	0·2	—	0·1	—	0·1	1	0·1	—	0·1	9	9·1
Liver	8	9·8	—	3·5	5	4·1	3	3·1	—	3·1	—	0·2
Bile passages	6	4·9	1	1·7	2	2·0	2	1·6	—	1·6	6	4·6
Pancreas	2	1·4	1	0·5	—	0·6	—	0·4	1	0·4	1	1·3
Peritoneum	2	1·6	—	0·6	—	0·7	—	0·5	1	0·5	3	1·5
Kidney	1	0·3	—	0·1	2	0·1	—	0·1	—	0·1	—	0·3
Bladder	—	1·1	—	0·4	—	0·4	2	0·3	—	0·3	—	1·0
Corpus uteri	3	4·7	—	1·6	2	1·9	2	1·5	2	1·5	3	4·3
Cervix uteri	14	6·7	12	2·4	2	2·8	1	2·1	5	2·1	12	6·2
Uterus n.o.s.	3	2·2	1	0·8	1	0·9	—	0·7	1	0·7	7	2·0
Vagina and vulva	1	1·1	—	0·4	1	0·4	—	0·3	—	0·3	—	1·0
Skin	—	0·9	—	0·3	—	0·4	1	0·3	2	0·3	1	0·8
Breast	19	19·3	9	6·8	6	8·0	4	6·1	6	6·1	24	17·9
Ovary	6	4·8	3	1·7	5	2·0	—	1·5	1	1·5	3	4·5
Other sites	7	3·9	—	1·3	—	1·6	2	1·4	3	1·4	5	3·6
Total	113	113	40	40	47	47	36	36	36	36	105	105

Site of carinoma.	*Wives of fishermen.*		*Wives of greengrocers.*		*Wives of Commercial travellers.*		*Wives of Market gardeners.*		*Wives of Navvies.*		*Wives of Hall porters.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	3	0·9	—	0·9	1	1·3	1	2·6	—	1·4	—	0·9
Stomach	10	12·6	10	13·1	16	19·0	55	37·7	18	20·1	5	13·9
Intestines	2	4·0	3	4·2	3	6·1	17	12·1	2	6·4	2	4·5
Rectum	2	2·1	1	2·2	2	3·2	7	6·3	2	3·3	3	2·3
Larynx	—	0·1	—	0·2	—	0·2	—	0·4	1	0·2	1	0·2
Lung	1	0·8	1	0·8	1	1·2	3	2·3	3	1·2	1	0·8
Tongue	—	0·1	—	0·1	—	0·2	—	0·3	—	0·2	—	0·1
Mouth	—	0·2	—	0·2	—	0·3	—	0·5	—	0·3	—	0·2
Jaw	—	0·2	—	0·2	—	0·3	—	0·5	1	0·3	—	0·2
Pharynx	—	0·1	—	0·1	—	0·1	—	0·2	—	0·1	—	0·1
Liver	5	4·1	3	4·2	2	6·2	8	12·2	14	6·5	2	4·5
Bile passages	1	2·0	—	2·1	—	3·1	3	6·1	4	3·3	2	2·3
Pancreas	—	0·6	1	0·6	2	0·9	1	1·7	1	0·9	1	0·6
Peritoneum	1	0·7	4	0·7	1	1·0	3	2·0	—	1·1	—	0·7
Kidney	—	0·1	—	0·1	1	0·2	—	0·4	—	0·2	—	0·1
Bladder	—	0·4	2	0·5	—	0·7	3	1·3	1	0·7	1	0·5
Corpus uteri	2	1·9	2	2·0	3	2·9	7	5·8	4	3·1	3	2·1
Cervix uteri	2	2·8	7	2·9	8	4·2	—	8·3	12	4·4	7	3·1
Uterus n.o.s.	—	0·9	1	0·9	—	1·4	2	2·7	2	1·4	—	1·0
Vagina and vulva	1	0·5	1	0·5	1	0·7	1	1·4	2	0·7	1	0·5
Skin	—	0·4	—	0·4	—	0·5	1	1·1	—	0·6	—	0·4
Breast	9	8·0	12	8·4	23	12·0	19	24·1	7	12·8	10	8·9
Ovary	6	2·0	—	2·1	4	3·0	5	6·0	1	3·2	7	2·2
Other sites	2	1·5	1	1·6	3	2·3	5	5·0	—	2·6	6	1·9
Total	47	47	49	49	71	71	141	141	75	75	52	52

TABLE XIII*a*—(*cont.*).

Site of carcinoma.	*Wives of Railway and Tram men.*		*Wives of Grocers.*	
	A.	E.	A.	E.
Oesophagus	—	3·4	1	1·6
Stomach	36	50·9	35	23·7
Intestines	13	16·3	7	7·7
Rectum	11	8·5	2	4·0
Larynx	1	0·6	—	0·3
Lung	4	3·1	—	1·5
Tongue	—	0·4	—	0·2
Mouth	—	0·7	—	0·3
Jaw	3	0·7	—	0·3
Pharynx	—	0·3	—	0·1
Liver	11	16·5	3	7·7
Bile passages	9	8·2	3	3·9
Pancreas	7	2·3	—	1·1
Peritoneum	7	2·7	1	1·3
Kidney	2	0·5	—	0·2
Bladder	2	1·8	—	0·8
Corpus uteri	2	7·8	4	3·7
Cervix uteri	16	11·2	7	5·3
Uterus n.o.s.	3	3·6	1	1·7
Vagina and vulva	1	1·8	—	0·9
Skin	—	1·4	—	0·7
Breast	42	32·5	21	15·2
Ovary	20	8·1	4	3·8
Other sites	6	6·7	—	3·0
Total	190	190	89	89

TABLE XIII*b*.—*Recorded and Expected Deaths from Carcinoma amongst Unmarried Women of Various Occupations.*

Site of carcinoma.	*Teachers.*		*Nurses.*		*Nuns and Religieuses.*		*Domestics (female), unmarried.*		*Needlewomen, unmarried.*	
	A.	E.	A.	E.	A.	E.	A.	E.	A.	E.
Oesophagus	4	1·8	—	1·0	4	3·3	8	7·3	—	2·7
Stomach	16	29·8	15	16·0	60	53·4	132	118·4	36	42·8
Intestines	12	11·0	3	5·9	19	19·8	34	43·9	15	15·9
Rectum	5	5·2	3	2·8	4	9·3	22	20·5	8	7·4
Larynx	—	0·4	—	0·2	—	0·7	3	1·4	1	0·5
Lung	2	2·2	5	1·2	3	4·0	11	8·9	3	3·2
Tongue	—	0·3	—	0·2	3	0·5	—	1·2	1	0·5
Mouth	—	0·2	—	0·1	—	0·3	—	0·7	—	0·2
Jaw	1	0·4	—	0·2	—	0·6	1	1·4	—	0·5
Pharynx	—	0·2	—	0·1	—	0·3	1	0·8	—	0·3
Liver	5	7·9	3	4·2	17	14·1	27	31·2	9	11·3
Bile-passages	1	1·8	2	1·0	5	3·3	12	7·2	2	2·6
Pancreas	—	0·9	1	0·5	2	1·6	5	3·6	2	1·3
Peritoneum	—	1·5	—	0·8	1	2·7	8	5·9	4	2·1
Kidney	—	0·3	—	0·2	—	0·5	—	1·2	—	0·5
Bladder	1	1·4	—	0·7	1	2·5	10	5·2	—	2·0
Corpus uteri	7	3·6	2	2·0	6	6·5	7	14·5	4	5·2
Cervix uteri	1	2·6	2	1·4	5	4·6	9	10·2	6	3·7
Uterus n.o.s.	6	2·6	1	1·4	3	4·6	5	10·2	1	3·7
Vagina and vulva	—	0·8	1	0·4	—	1·5	1	3·3	—	1·1
Skin	1	1·2	—	0·6	—	2·2	4	4·9	—	1·8
Breast	34	24·1	13	12·9	44	43·1	95	95·6	34	34·6
Ovary	8	6·1	8	3·3	11	10·9	29	24·3	16	8·8
Other sites	6	3·8	—	1·9	9	6·7	13	15·2	6	5·3
Total	110	110	59	59	197	197	437	437	158	158

Note.—A. = Number of deaths actually recorded.
E. = Number of deaths expected from the site distribution of carcinoma deaths amongst all single women.

The total mortality from carcinoma for the different trades and professions, from age-classes 40–49 and 50–59 only, is shown in Table XIV as percentages of the number to be expected.

TABLE XIV.—*Total Mortality as Percentages of Expected Number.*

	Per cent.		Per cent.
Publicans, etc.	200	Diamond-cutters	100
Headteachers	91	Firemen	61
Butchers	161	Fishermen	194
Bakers	102	Greengrocers	220
Smiths	69	Navvies	62
Shoemakers	100	Hall-porters	100
Farmers	92	Cigar-manufacturers and makers	155
House-painters	100	Tobacconists	121
Weavers	140	Domestics (females)	72
University-men	100	Needlewomen	129
Office-staffs	97	Plumbers	225
Tailors	100	Printers	117
Masons	91	Grocers	106
Bargemen	90	Police	79

It is quite evident that there is not an equal tendency among all people to be affected by carcinoma, and this shows itself in a distribution by organs typical for each trade. The total mortality is the sum of the carcinoma mortalities of all organs.

In those trades in which the total mortality is 100 per cent—equal to that of the average population—a relatively increased frequency for a particular organ also means an absolute increase in the carcinoma frequency for that organ. But in those trades in which the total mortality is much higher or lower than 100 per cent, a relative increase or decrease need not necessarily mean a higher or lower absolute carcinoma frequency. In these cases the second method, working from the age-classes 40–49 and 50–59, has a correcting influence.

For instance, whereas we expected 180 cases of carcinoma of the stomach among publicans, etc., we actually found only 119. This would seem to be a reduced incidence, but if we look at the numbers for the age-groups 40–49 and 50–59 we find that the incidence is actually increased—37 instead of the expected 30.

In those trades where the total frequency differs markedly from that of the general population we should calculate the number of carcinomas to be expected in each organ, not by distributing the number of cases found for the trade over the different organs according to the average distribution for the general population, but by distributing $\frac{100}{x}$ times that number found over the different organs, where x is the total mortality for the trade expressed as a percentage of the number to be expected as shown in Table XIV.

Thus we have found that for publicans, etc., the total mortality from carcinoma is 200 per cent that of the general population. If in accordance with the proportions in the general population we distributed the actual number of cases of carcinoma over the various organs, we should expect 180 cases of carcinoma of the stomach. But we have distributed double the number of cases that

would have been expected on the basis of the general population. Accordingly we should not compare the 119 cases found with 180, but only with 90, which is the number of carcinomas of the stomach to be expected among publicans at ages between 40 and 60 if they had the same frequency as in the average population.

Working in accordance with both these methods we get the results shown in Table XV, and by comparing the figures for the men and their wives we can tell whether the excess found for an organ depends on the occupation or on the environment.

TABLE XV.—*Organs Affected by Carcinoma in Excess or Defect of Expectation in Different Occupations.*

Occupation group.	*Increased.**	*Probably† increased.*	*Probably† reduced.*	*Reduced.**	*Comments.*
Farmers	stomach skin	lip	..	intestines larynx lung tongue pancreas	Farmers' wives also have stomach excess.
Agricultural labourers	stomach skin	..	pancreas kidney tongue	rectum larynx lung	Among farmers are many who are no more than agricultural labourers.
Stock farmers	stomach	..	intestines	bladder	..
Market gardeners	..	..	lung	..	..
Greengrocers	stomach	liver	..	..	..
Fishermen	oesophagus stomach jaw	liver skin peritoneum	..	..	In Holland the fishermen's wives mend the nets, consequently no such excess of lip carcinoma as in England where the needles are put in the mouths.
University men	lung pharynx prostate	tongue	..	stomach	Clergymen have much prostate and physicians much lung carcinoma.
Masters (Grammar-schools, etc.)	lung prostate	intestines penis	..	stomach	..
Teachers	intestines	liver	..	stomach	Lady teachers show no increase for intestine.
Office staffs	lung intestines	mouth pharynx	..	stomach	Are always in smoky environment.
Publicans, Hotel keepers, Waiters, Coffeehouse keepers	oesophagus larynx lung tongue mouth pharynx bladder	stomach intestines rectum liver pancreas prostate	..	..	Is this an indication of the possibility of a general disposition for carcinoma?
Soldiers	larynx	tongue liver prostate	..	stomach	The Indian soldiers have no increased liver-frequency. Dutch land-forces and English Navy quite agree.

* i.e. by three or more times the standard error.
† "Probably" means: the increase or reduction is twice, but not three times, the standard error.

TABLE XV—(*cont.*).

Occupation group.	*Increased.**	*Probably† increased.*	*Probably† reduced.*	*Reduced.**	*Comments.*
Railwaymen, etc.	..	lip jaw	skin	..	..
Firemen	..	..	oesophagus intestines rectum	stomach	..
Smiths	penis	jaw	lung oesophagus liver	stomach	..
Commercial travellers	lung mouth	oesophagus pancreas	..	..	..
Butchers	lung bladder	stomach intestines prostate	..	..	Any connection between lung and echinococcus? In my opinion wrong diagnosis is not probable; liver carcinoma is not increased either.
Tailors	prostate	penis	..	..	..
Drapers	lung	rectum larynx kidney	..	stomach	..
Carpenters	..	penis	..	..	..
Masons	..	..	..	..	..
Grocers	..	lung	..	..	..
Shoemakers	..	mouth bladder	..	..	Skin: not increased. Is bladder due to aniline?
Diamond cutters	lung	..	..	..	..
Police	..	rectum lip	stomach	..	..
Hall porters	lung	mouth	..	..	Smoke in closed space.
Cigar makers and cigar manufacturers	stomach lung	prostate	..	..	..
Tobacconists	lung	tongue	..	..	Smoke in closed space.
Plumbers	..	liver pancreas	..	..	Lead?
Printers	..	intestines	..	..	Lead?
Weavers	stomach liver	..	..	..	Is liver-carcinoma a trade illness? See Weavers' wives.
Carmen	bladder	pharynx	intestines	..	..
Dock labourers	..	tongue	..	..	..
Navvies	..	..	intestines	lung prostate	*Oil venders* are only a small group but have an excessively high stomach frequency (26 of 35 carcinomas).
Bargemen	..	skin	..	..	
General labourers	oesophagus	penis	..	lung	
Miners	stomach	..	..	prostate	
		Wives of—			
Farmers	stomach liver	..	bladder uterus	cervix uteri	..
Farmers' labourers	oesophagus stomach	..	intestines corpus uteri ovary	cervix uteri	Despite many children, few cervix carcinomas, so cervix rupture is of secondary importance.
Stock farmers	..	ovary	cervix uteri	..	..

TABLE XV—(*cont.*).

Occupation group.	*Increased.**	*Probably† increased.*	*Probably† reduced.*	*Reduced.**	*Comments.*
		Wives of—			
Market gardeners	..	stomach	cervix uteri	..	..
Greengrocers	..	cervix uteri	..	..	..
Fishermen	..	ovary	..	..	..
University men	ovary	..	liver	stomach	..
Teachers	breast	..	..	..	..
Office staffs	breast ovary	intestines	..	stomach	..
Publicans	..	cervix uteri breast	ovary	..	..
Railwaymen	ovary	peritoneum pancreas	stomach	..	..
Firemen	..	cervix uteri	stomach	..	..
Commercial travellers	breast	cervix uteri	..	..	..
Butchers	cervix uteri	..	..	..	..
Masons	..	rectum	..	..	..
Dealers in fuel	..	..	liver	..	..
House painters	..	cervix uteri breast	..	..	..
Shoemakers	..	stomach	..	..	..
Police	cervix uteri	..	..	..	..
Hall porters	ovary	cervix uteri	stomach	..	..
Weavers	..	liver	breast	..	..
Navvies	cervix uteri	liver	..	..	..
Bargemen	tongue cervix uteri	..	stomach	..	..
General labourers	..	skin	..	..	..
Lady teachers	..	breast	stomach	..	..
Nurses	lung	ovary	..	..	Is the lung carcinoma dependent on ether or on a better diagnosis?
Domestics (females)	..	..	liver uterus (corpus and unspecified) breast	intestines	..
Needlewomen	ovary	..	..	..	..

When arranged according to the *organs*, we find excess or deficiency of carcinoma for the following groups of men :

Organ.	*Increased.*	*Probably increased.*	*Probably reduced.*	*Reduced.*
Oesophagus	Barmen General labourers Fishermen	Commercial travellers	Firemen Smiths	..
Stomach	Fishermen Weavers Farmers Agricultural lab. Stock farmers St. Farmhands Greengrocers Cigar makers Miners	Barmen Butchers	Police	Firemen Drapers Smiths. Soldiers. University men. Masters. Office staffs. Teachers

TABLE XV—(*cont.*).

Organ.	*Increased.*	*Probably increased.*	*Probably reduced.*	*Reduced.*
Lung	Drapers Barmen Butchers University men Masters Office staffs Diamond cutters Comm. travellers Hall porters Cigar makers Tobacconists	Grocers	Smiths Market gardeners	Farmers. Agricultural lab. General lab. Navvies.
Larynx	Barmen Soldiers	Drapers	..	Farmers. Agricultural lab.
Intestines	Teachers Office staffs	Barmen Butchers Masters Printers	Carmen Firemen Stock farmers Navvies	Farmers.
Rectum	..	Barmen Drapers Police	Firemen	Agricultural lab.
Bladder	Butchers Barmen Car men	Shoemakers	..	Stock farmers.
Prostate	University men Masters Tailors	Barmen Butchers Soldiers Cigar makers	.. ..	Navvies. Miners.
Tongue	Barmen	Soldiers University men Tobacconists Dock labourers	Agricultural lab.	Farmers.
Mouth	Barmen Commer. travellers	Shoemakers Office-staff Hall porters	..	..
Pharynx	Barmen University men	Office staffs Car men	..	..
Liver	Weavers	Barmen Teachers Soldiers Fishermen Plumbers Greengrocers	Smiths	..
Pancreas	..	Barmen Plumbers Commer. travellers	Farmers Agricultural lab.	..
Peritoneum	..	Fishermen	..	..
Penis	Smiths	Carpenters General labourers Masters Tailors	..	..
Jaw	Fishermen	Smiths Railwaymen	..	..
Lip	..	Farmers Market gardeners Railway men Police	..	..
Skin	Farmers Agricultural lab.	Bargemen Fishermen	Railwaymen	..
Kidney	..	Drapers	Agricultural lab.	..

Amongst *wives* of those who carry on the different trades, and *women* in female trades, we find excess or deficiency for the following organs:

TABLE XV—(*cont.*).

Organs.	*Increased.*	*Probably increased.*	*Probably reduced.*	*Reduced.*
Stomach	Farmers Farmers' labourers	Market gardeners Shoemakers	Railwaymen Bargemen Firemen Hall porters Lady teachers	University men. Office staff.
Oesophagus	Farmers' labourers	..	..	..
Lung	Nurses	..	..	..
Intestines	..	Office staff	Farmers' labourers	Domestics (females).
Rectum	..	Masons	..	..
Bladder	..	..	Farmers	..
Tongue	Bargemen	..	..	..
Liver	Farmers	Navvies	University men Dealers in fuel Domestics (female)	..
Pancreas	..	Railwaymen	..	..
Peritoneum	..	Railwaymen	..	..
Cervix uteri	Police Butchers Bargemen Navvies	Publicans Painters Firemen Greengrocers Commer. travellers Hall porters	Market gardeners Stock farmers	Farmers Farmers' lab.
Breast	Teachers Office staff Commer. travellers	Lady teachers Publicans Painters	Weavers Domestics (female)	..
Ovary	Railwaymen Office staff Hall porters University men Needlewomen	Stock farmers Fishermen Nurses	Farmers' labourers Publicans	..
Uterus (unspecified)	..	..	Domestics Farmers	..
Corpus uteri	..	..	Farmers' labourers Domestics	..
Skin	..	General labourers	..	..

CONCLUSIONS.

It would be interesting to consider what conclusions may be drawn from the figures in this paper, but that would take up too much space. Besides, in my opinion conclusions will only be justifiable when we have sufficient international material at our disposal for comparison.

This material will have to consist of the results of research on many, relatively small, areas during a period shortly before the Second World War, with due observance of the necessary precautions. With larger areas racial, geographical and economic differences in the same trade play a prominent part, and a trade may be quite different in one country from another. During and after the war there was too much changing of occupation.

I wish to express my gratitude to Dr. W. F. Wassink of the Antonie van Leeuwenhoekhuis, Amsterdam, for his interest in this work and for his encouragement. Many thanks are also due to Sir Percy Stocks, for all the help he gave me, particularly in connection with the statistics.

THE MALIGNANCY OF CANCER AT DIFFERENT AGES: A HISTOLOGICAL STUDY.

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THERE is a general impression among clinicians and pathologists that the malignancy of cancer is greater in relatively younger people, and there is certainly a strong belief that cancer occurring in the very old may be so slow in growth as not to limit the "natural" duration of life.

In an attempt to find out to what extent these impressions are justified we shall give:

(*a*) The opinions put forward in some relevant articles in the literature.

(*b*) The results of a study of the microscopic structure of some tumours in patients of different age groups.

Most diseases have an age period of maximum frequency and probably also an age period of maximum intensity and virulence. It does not follow, of course, that these age periods coincide, although it would be reasonable to expect that the age group in which the disease occurred most frequently was also that in which the disease occurred most virulently.

Cancer is predominantly a disease of senescence, and if one had, *a priori*, to foretell its malignancy, one would expect its virulence to be most intense at higher age periods.

On the other hand, the cancer cell seems to differ from the physiological cell only in its greatly increased growth potential. It seems otherwise to share, perhaps disproportionately, in the metabolic turnover of the body. One might expect, since the nutritive efficiency is presumptively greater in the young, that the cancer cells would grow and divide rather faster in the young.

In actual fact the age incidence of disease by frequency and by virulence has not much law or reason, and is just an empirical fact.

We have little doubt that in so far as the belief in increased virulence of cancer at young ages is not based on clinical experience it is based on the assumption that physiological tissue growth and activity is much greater in the young, as, for example, in the muscular hyperplasia following functional over-use.

RELEVANT LITERATURE.

The malignancy of any tumour can be measured clinically in two ways—by the duration of the untreated disease, and by the curability rate after standard treatment.

Opportunities for studying the first are fortunately diminishing, and one is largely dependent on the classical work of Lazarus-Barlow and Leeming (1924) and of Wyard (1925). Greenwood (1926) gave tables of figures from the data of

these and other workers showing the mean natural duration by age groups for untreated cancer of breast, cervix uteri, rectum, larynx, oesophagus, stomach, tongue and mouth (males). He commented (p. 15):

> "It will be seen that either there is no significant relation between age at onset and duration or, at the least, these data are not numerous enough to establish it";

and again (p. 15):

> " . . . on the testimony of these data we cannot confirm the general opinion of clinicians that age at onset is an important factor of duration" (i.e. duration of life with untreated cancer).

There are two other fairly large series of cases of untreated cancer.

Daland (1927) described the course of 100 cases of untreated cancer of the breast. At one place (p. 266) he said that "cancer of the breast is usually very malignant in a young woman," but his figures show no definite correlation between duration of survival and age.

Daland, Welch and Nathanson (1936) analysed the findings in 100 cases of untreated rectal cancer and tabulated their results. They commented (p. 453):

> "It will be observed from the table that the age of the patient bears no relation to the duration of the disease."

Some opinions regarding age-malignancy correlations in different series of treated carcinomas at varying sites are given below.

Breast.

Sistrunk and MacCarty (1922, p. 62):

> "Age seems to have a definite bearing on the results to be expected following operation. Forty-one and seven-tenths per cent of the patients over fifty are alive from five to eight years after operation, while only 31·8 per cent of those under fifty have lived a corresponding time."

Stout (1932, p. 301):

> "For the large majority of breast cancers the age of the woman has little significance, but for the extremes prognosis is definitely affected in group statistics by the age at time of operation. In our experience below the age of forty years axillary metastasis is much more apt to be present at the time of operation than beyond that age. In the same way postoperative survivals, as a whole, and apparently cancer-free survivals are decidedly fewer below the age of thirty-five years and decidedly greater beyond the age of sixty-nine years."

Nathanson and Welch (1936, p. 52):

> "The median length of life in the age group below forty is about three years, from forty to sixty about three and a half years, and about four years thereafter. Cancer of the breast in the hospital cases is more malignant in the young and less so in the old."

Scarff and Handley (1938, p. 583):

> "It has been current teaching that carcinoma occurring in a young woman kills more rapidly than in an old woman. Our investigation has not borne

this out and Table III shows that age has little bearing on prognosis. No significant variation has been found in the proportion of cases showing axillary involvement in the various age-groups."

Macdonald (1942, p. 76):

"The evidence presented here strongly supports the contention that the best results in the treatment of breast cancer are obtained between the ages of 35 and 50. The thesis that an especial virulence exists for mammary carcinoma in the young is not verified."

Haagensen and Stout (1942) studied the data in 1040 cases of carcinoma of the breast. In 640 of these cases radical mastectomy was carried out. Of these 640 patients, 18 were aged less than 30 years. These authors commented (p. 860):

"These data leave no doubt that radical mastectomy is worth while, even in the youngest patients with breast carcinoma, provided, of course, that the lesion itself is not too far advanced. If the three patients under the age of 30 in our series whose tumor developed during pregnancy or lactation are excluded . . . the five-year cure rate in the remaining 15 young patients rises to 37·5 per cent, a figure quite comparable with the cure rate for our series of cases as a whole.

"Indeed, when the patients in our series are grouped according to age into broader categories there is surprisingly little difference in the results of operation in the different groups." (The age groups were, Under 45, 45 to 49, and 50 or over.)

de Cholnoky (1943, p. 60):

"The conclusion is reached that the results of radical surgery in young women under 30 years of age are comparable to those obtained in the more advanced age groups. The previously held belief that the prognosis for women under 30 years of age who have malignant tumors of the breast is fatal seems untenable."

Hawkins (1943, p. 456):

"Although it is difficult to weigh properly all the various dependent variables involved, the age of the patient does not significantly influence the over-all results of breast-cancer therapy."

Geschickter (1945, p. 404):

"Thus, while the number of five-year survivals in the younger age group compares favorably with those in the older group, the average duration of life for both cured and non-cured patients is less in the younger group. This is because of the rapid termination of fatal tumors in the young, particularly during pregnancy and lactation."

Rectum.

Fowler (1926, p. 77):

"It cannot be said, therefore, that the presence or absence of involvement of the lymph nodes in rectal carcinoma in the young is of any prognostic value as far as recovery is concerned. However, a higher percentage of

involvement of lymph nodes is found, and the average postoperative life is shorter (16·7 months) than in similar cases in the adult."

Dukes (1940) found that patients under the age of 40 years had a higher incidence of lymphatic metastases than those in the age group 40–59 years. He commented (p. 529):

"The most likely explanation is that cancer of the rectum gives rise to metastases more rapidly in young patients than in old."

Uterus (cervix).

Lane-Claypon (1927, p. 34):

"The evidence therefore does not show any tendency to greater virulence or apparently to a more rapid dissemination of the growth in youth."

Lung.

Geschickter and Denison (1934) analysed 60 cases of primary lung cancer and graded them histologically, correlating the histological findings with clinical malignancy and with age. They gave their opinion that the most malignant forms, both clinically and histologically, tended to occur at the younger ages.

Frissell and Knox (1937) gave details of 46 cases of primary lung cancer which included the age and the duration of the disease for each patient. On examining these data we have found that there is no relation at all between age and the duration of the disease. The authors stated (p. 225):

"These figures are sufficient to indicate the rarity of carcinoma of the lung under the age of forty. It is, however, characterized by the same clinical and pathological course in this age group as in more elderly persons."

The following opinions have been put forward more particularly with reference to malignancy as assessed histologically.

Carcinoma in General.

Fowler (1926) analysed the findings in 112 cases of malignant epithelial neoplasms occurring in persons under 26 years of age. He divided them into a "carcinoma" group of 89 patients and an "epithelioma" group of 23 patients. He had no doubt that the clinical malignancy of the tumours in the carcinoma group was greater in these patients than in older patients, and he analysed the histological findings to see whether the histological malignancy ran parallel. He stated (p. 74):

"Compared with sections of similar tumors in older persons, the tumors in these cases give a microscopic picture of a more rapidly growing, undifferentiated tissue, often with many mitotic figures in each field."

He found that hyalinization was never present; that lymphocytic infiltration and fibrosis were present to a moderate degree and only in the less malignant tumours; and that cellular differentiation was present mainly in ovarian and thyroid carcinomas where, in each instance, the mortality was comparatively low. He concluded (p. 83):

"The lack of hyalinization, fibrosis, lymphocytic infiltration, and cellular differentiation may have been responsible for the increased malignancy of these neoplasms in the young."

Rectum.

Dukes (1940) divided his cases of rectal carcinoma into histological grades. He found (p. 532):

> "The distribution of the grades was approximately the same in each sex, but tumours of high-grade malignancy appeared to be more common in young patients, though the difference did not quite reach the accepted level of significance in the χ^2 test."

Lung.

As mentioned earlier, Geschickter and Denison (1934) analysed 60 cases of primary lung carcinoma. One of their conclusions was (p. 875):

> "Hilar or epidermoid carcinoma may be graded into three forms. In the least malignant histological grade, occurring in patients over fifty years, squamous cells predominate. The middle grade shows a proliferation of basal and transitional cells and occurs in patients under fifty years. The most malignant grade shows masses of compact or spindle cells, is referred to as oat-cell cancer, and is most common in young adults. Lobular adenocarcinoma is also divided histologically into three groups. The least malignant or most highly differentiated tumors are of two forms, adenocolumnar and adenomucoid. The average age of these patients is forty years. The most malignant histologic type of adenocarcinoma shows a diffuse proliferation of cuboidal cells (adenocuboidal cancer). The patients affected are most often under forty years of age and sometimes under thirty."

Frissell and Knox (1937) gave the histological diagnosis of the type of growth in all but three of their 46 cases of primary lung carcinoma. Analysis of their data shows no significant association of the least differentiated tumours with the younger patients.

Stomach.

With regard to the 9 cases of gastric carcinoma included in his series, Fowler (1926) commented (p. 78):

> "The histological picture is that of a rapidly growing adenocarcinoma, the cells in some parts retaining their glandular arrangement, in others forming long cords or solid groups of undifferentiated cells. . . .
>
> "This pathological appearance is borne out by the fact that death occurred within six months of the date of operation in all cases."

It will be seen from these quotations that opinions vary on the subject of the relation of age to both clinical and histological malignancy. Certainly for the breast opinion seems to have veered during the last decade to a denial of increased malignancy at younger ages.

Ewing (1940) commented on the matter as follows (p. 17):

> "The weight of opinion indicates that adult types of tumors occurring at early ages tend to run a more rapid course, to metastasize more widely, but this view is contested by many observers."

Since there is such diversity of opinion as to whether this effect exists at all it is reasonable to assume that if it does exist it is small.

ASSESSMENT OF MICROSCOPIC APPEARANCES OF CARCINOMAS AT DIFFERENT AGES.

Our material has been taken from the collection of microscopic sections of tumours in this laboratory.

For many years it has been the rule that when a neoplasm was diagnosed during routine reporting it was recorded in classifications by site and by type. It frequently happened, of course, that the diagnosis was in doubt. When this happened the tumour was recorded once, and once only, under its most likely diagnosis although a note of it might be made for reference under several headings.

Since there is no reason to suspect that there was any systematic bias towards diagnosing malignancy unduly at one age group rather than another, comparison of random samples of the tumour sections from different age groups is statistically valid.

Procedure.

We went through the index cards of the years 1930 to 1948. Whenever a case of carcinoma was found occurring at age 30 or under in any of uterus, lip, lung, stomach, colon, rectum or breast, we carried on through the cards and chose the first carcinoma recorded in the same organ and of the same sex in an individual in age group 46–55 and again in a high age group. Due to the varying rarity of cancer at high ages the high age groups chosen were :

75 years and over for breast ;
70 years and over for uterus, lip, stomach, colon, rectum ; and
61 years and over for lung (where the middle age group was 41–50).

For each tumour type we thus had three randomly chosen groups of slides, of equal numbers, occurring in the young, the middle-aged and the old. The slides were then taken out of their holders and shuffled. This made identification of the sections impossible without reference to the laboratory register of the numbers etched on the slides. The shuffled and randomized sections were then examined for some or all of certain characteristics :

Degree of epidermoid or glanduliform differentiation.
Degree of fibrosis.
Cell size and polymorphism.
Amount of " colloid " formation.
Mitotic frequency.
General impression of malignancy.

All these features were given one of four grades of increasing expression.

For *cell size* (which in this context means cell largeness), *cell polymorphism* and *degree of fibrosis* (scirrhous reaction) the principle adopted was to allocate Grades I and IV to cases which were strikingly low and strikingly high respectively in these features. Grades II and III formed intermediate groups of " low moderate " and " high moderate " respectively.

For *epidermoid differentiation* the scale, as well as could be defined, was :

Grade I. No differentiation.
,, II. Formation of solid cell masses with a demarcated cellular border, i.e. a " solid alveolar " pattern.
,, III. Formation of prickle cells and squamous cells.
,, IV. Formation of definite keratinized " pearls."

Glanduliform differentiation varied from no differentiation in Grade I, upwards, with increasingly glanduliform pattern, through Grades II, III and IV.

Carcinomas of colon and rectum usually show relatively much glandulifrom differentiation. At these sites Grade I meant no or notably little glanduliform differentiation.

"Colloid" formation.—In this feature Grade I applies to cases showing no "colloid" formation. Grades II, III and IV indicate increasing proportions of this degeneration.

Mitotic frequency.—Tumour types tend to have a characteristic mitotic frequency. The gradings used for the various organs were:

	Uterus, rectum, colon.	Breast, stomach.	Lip.
Grade I	0–1	0–1	0–2
,, II	2–3	2	3
,, III	4–5	3	4
,, IV	6 and +	4 and +	5 and +

In each case the figures refer to the average number of mitoses per 1/6 in. field. In scirrhous tumours allowance was made for the space occupied by fibrous tissue.

When all the sections had been examined and the various factors assessed the slides were identified by reference to the laboratory register and the results then tabulated according to the three age groups. The results are shown in Table I.

TABLE I.—*Distinguishing Age, Site of Carcinoma and Grade of Degree, Intensity or Extent of Each Feature Estimated. (The figures give the number of cases occurring within each group.)*

	Number of cases.		Epidermoid differentiation.				Glanduliform differentiation.				Scirrhous reaction.			
	M.	F.	I.	II.	III.	IV.	I.	II.	III.	IV.	I.	II.	III.	IV.
Age 30 years or less.														
Carcinoma of—														
(*a*) Uterus	..	20*	3	7	6	0	1	2	1	0	5	3	11	21
(*b*) Lip	9	1	0	0	5	5	..	..	..	..	..	..	..	..
(*c*) Lung	20	2	14	6	2	0	21	0	1	0	..	..	..	..
(*d*) Stomach	2	6	..	..	..	..	4	4	0	0	0	1	6	1
(*e*) Colon	3	3	..	..	..	..	2	1	2	1	1	2	3	0
(*f*) Rectum	7	7	..	..	..	..	3	5	5	1	1	4	4	5
(*g*) Breast	..	25	..	..	..	..	16	1	7	1	11	11	3	0
Total	41	64	17	13	13	5	47	13	16	3	18	21	27	27
Age 46–55 years.														
Carcinoma of:														
(*a*) Uterus	..	20*	1	8	5	2	0	1	3	0	4	4	9	3
(*b*) Lip	9	1	0	2	4	4	..	..	..	..	..	..	..	..
(*c*) Lung (41–50 yrs.)	20	2	7	6	8	1	20	2	0	0	..	..	..	..
(*d*) Stomach	2	6	..	..	..	..	3	2	2	1	1	2	4	1
(*e*) Colon	3	3	..	..	..	..	0	2	4	0	0	1	5	0
(*f*) Rectum	7	7	..	..	..	..	0	3	8	3	1	4	9	0
(*g*) Breast	..	25	..	..	..	..	16	4	5	0	8	8	9	0
Total	41	64	8	16	17	7	39	14	22	4	14	19	36	4
Age 70 years or more.														
Carcinoma of:														
(*a*) Uterus	..	20*	6	7	1	2	0	1	1	2	6	1	12	1
(*b*) Lip	9	1	1	3	5	1	..	..	..	..	..	..	..	..
(*c*) Lung (61 y. and over)	20	2	8	3	7	4	22	0	0	0	..	..	..	..
(*d*) Stomach	2	6	..	..	..	..	1	1	3	3	2	5	1	0
(*e*) Colon	3	3	..	..	..	..	2	0	3	1	0	0	5	1
(*f*) Rectum	7	7	..	..	..	..	0	4	5	5	1	3	9	1
(*g*) Breast (75 y. and over)	..	25	..	..	..	..	15	3	4	3	13	6	6	0
Total	41	64	15	13	13	7	40	9	16	14	22	15	33	3

* 16 cervical and 4 corporeal carcinomas.

TABLE I—(*cont.*).

	Number of cases.		Cell size.				Cell polymorphism.				Mitotic frequency.			
Age 30 years or less.	M.	F.	I.	II.	III.	IV.	I.	II.	III.	IV.	I.	II.	III.	IV.
Carcinoma of:														
(*a*) Uterus	..	20*	..	..	..	..	..	..	..	..	4	12	4	0
(*b*) Lip	9	1	..	..	..	..	..	..	..	..	8	1	0	1
(*c*) Lung	20	2	6	11	4	1	2	11	8	1	..	..	..	..
(*d*) Stomach	2	6	..	..	..	..	..	..	..	..	3	3	2	0
(*e*) Colon	3	3	..	..	..	..	..	..	..	..	2	3	1	0
(*f*) Rectum	7	7	..	..	..	..	..	..	..	..	5	7	2	0
(*g*) Breast	..	25	1	8	13	3	..	..	..	..	8	9	6	2
Total	41	64	7	19	17	4	2	11	8	1	30	35	15	3
Age 46–55 years.														
Carcinoma of:														
(*a*) Uterus	..	20*	..	..	..	..	..	..	..	..	9	7	4	0
(*b*) Lip	9	1	..	..	..	..	..	..	..	..	8	0	2	0
(*c*) Lung (41–50 y.)	20	2	3	9	10	0	1	14	5	2	..	..	..	..
(*d*) Stomach	2	6	..	..	..	..	..	..	..	..	1	5	2	0
(*e*) Colon	3	3	..	..	..	..	..	..	..	..	1	2	3	0
(*f*) Rectum	7	7	..	..	..	..	..	..	..	..	3	6	5	0
(*g*) Breast	..	25	0	9	15	1	..	..	..	..	9	11	1	4
Total	41	64	3	18	25	1	1	14	5	2	31	31	17	4
Age 70 years or more.														
Carcinoma of:														
(*a*) Uterus	..	20*	..	..	..	..	..	..	..	..	14	5	1	0
(*b*) Lip	9	1	..	..	..	..	..	..	..	..	5	4	1	0
(*c*) Lung (61 y. and over)	20	2	2	8	12	0	4	9	8	1	..	..	..	..
(*d*) Stomach	2	6	..	..	..	..	..	..	..	..	1	3	1	3
(*e*) Colon	3	3	..	..	..	..	..	..	..	..	4	2	0	0
(*f*) Rectum	7	7	..	..	..	..	..	..	..	..	3	4	6	1
(*g*) Breast (75 y. and over)	..	25	0	13	9	3	..	..	..	..	12	8	4	1
Total	41	64	2	21	21	3	4	9	8	1	39	26	13	5

	Number of cases.		"Colloid" formation.				General malignancy.			
Age 30 years or less.	M.	F.	I.	II.	III.	IV.	I.	II.	III.	IV.
Carcinoma of:										
(*a*) Uterus	..	20*	..	..	..	..	1	4	13	2
(*b*) Lip	9	1	..	..	..	..	3	6	0	1
(*c*) Lung	20	2	..	..	..	..	1	6	11	4
(*d*) Stomach	2	6	7	0	1	0	0	4	4	0
(*e*) Colon	3	3	2	0	2	2	0	3	3	0
(*f*) Rectum	7	7	8	2	4	0	1	5	5	3
(*g*) Breast	..	25	25	0	0	0	4	7	11	3
Total	41	64	42	2	7	2	10	35	47	13
Age 46–55 years.										
Carcinoma of:										
(*a*) Uterus	..	20*	..	..	..	..	6	8	5	1
(*b*) Lip	9	1	..	..	..	..	4	5	1	0
(*c*) Lung (41–50 y.)	20	2	..	..	..	..	2	14	5	1
(*d*) Stomach	2	6	8	0	0	0	0	3	5	0
(*e*) Colon	3	3	6	0	0	0	0	1	5	0
(*f*) Rectum	7	7	14	0	0	0	1	6	6	1
(*g*) Breast	..	25	25	0	0	0	3	10	10	2
Total	41	64	53	0	0	0	16	47	37	5
Age 70 years or more.										
Carcinoma of:										
(*a*) Uterus	..	20*	..	..	..	..	5	7	6	2
(*b*) Lip	9	1	..	..	..	..	1	6	2	1
(*c*) Lung (61 y. and over)	20	2	..	..	..	..	3	10	7	2
(*d*) Stomach	2	6	8	0	0	0	1	1	6	0
(*e*) Colon	3	3	5	0	1	0	1	2	2	1
(*f*) Rectum	7	7	11	0	3	0	1	5	8	0
(*g*) Breast (75 y. and over)	..	25	23	0	2	0	7	9	8	1
Total	41	64	47	0	6	0	19	40	39	7

* 16 cervical and 4 corporeal carcinomas.

Analysis of the Tables.

We started this experiment with the hypotheses:

(*a*) That there was no correlation between age and any of the factors examined.

(*b*) That if there was a correlation between age and any factor we had no reason to expect the correlation with one organ or one factor more than another.

The analysis of the figures therefore resolves itself into an estimation of the probability with which these figures would occur by chance if these hypotheses were true.

The collection of the data was designed so that we should be able to extract the effects due to (*a*) age (in three blocks—youth, middle age, old age), and (*b*) any change in the threshold for diagnosis of malignancy or in the type of material sent to the laboratory between 1930 and 1948, the period covered by the material.

This was done by balancing each unit in age group 30 and under by a randomly selected unit in each of the other age groups diagnosed at about the same time. After completing the experiment, we were quite convinced that there was no such gross systematic change in type of material or standards of diagnosis over the years. We have, therefore, not considered that the difference between blocks arranged by date of diagnosis would appreciably reduce the residual error on which the significance of inter-age differences would be estimated.

The experiment was therefore designed to allow for an analysis of variance. For example, in the sub-experiment "degree of glanduliform differentiation in rectal carcinoma" there was a total of 42 units or slides divided into 3 groups of 14 each for patients of 30 years or less, 46 to 55 years, and 70 years or more respectively, and divisible also into 14 groups of 3 each, 1 in each age group, diagnosed in the same calendar year. In an analysis of the variance in this balanced design there would then have been a total of 41 "degrees of freedom" comprising 2 for age, 13 for date of diagnosis, and 26 for residual error. Of the 2 degrees of freedom for age, one could have been used to test the linearity of the difference by age if necessary. In fact there was no need for this since a relatively coarse analysis of the data indicated that the figures might well have occurred by chance —on the hypothesis that there was no correlation between age and any of the factors we estimated.

The χ^2 test was carried out for each factor, with the exception of "'colloid' formation" and "cell polymorphism," using the total figures of each factor for each age group. For example, in the case of "epidermoid differentiation" there was a contingency table of the form—

17	13	13	5
8	16	17	7
15	13	13	7.

The results for the various factors were:

Epidermoid differentiation:

$$n = 6 \quad \chi^2 = 4{\cdot}95 \quad P = 0{\cdot}7\text{–}0{\cdot}5.$$

Glanduliform differentiation (consolidating Grades III and IV):

$$n = 4 \quad \chi^2 = 4{\cdot}55 \quad P = \text{c. } 0{\cdot}3.$$

Scirrhous reaction (consolidating Grades III and IV):

$$n = 4 \quad \chi^2 = 3{\cdot}30 \quad P. = c.\ 0{\cdot}5.$$

Cell size (consolidating Grades I and II, and Grades III and IV):

$$n = 2 \quad \chi^2 = 1{\cdot}08 \quad P = 0{\cdot}7\text{–}0{\cdot}5.$$

Mitotic frequency (consolidating Grades III and IV):

$$n = 4 \quad \chi^2 = 3{\cdot}12 \quad P = c.\ 0{\cdot}5.$$

General malignancy:

$$n = 6 \quad \chi^2 = 10{\cdot}06 \quad P = 0{\cdot}2\text{–}0{\cdot}1.$$

It will be seen that in no case are the figures "significantly" different from what might be expected by chance. Inspection of Table I does indeed suggest that the figures for epidermoid differentiation in bronchogenic carcinoma might well give a significant correlation between increasing age and increasing degree of epidermoid differentiation. We have carried out a χ^2 test for epidermoid differentiation in bronchogenic carcinoma alone consolidating the figures for Grades II, III and IV. This gives $n = 2$ $\chi^2 = 5{\cdot}29$ $P = 0{\cdot}05$ ($P_{\cdot 05} = 5{\cdot}991$ $P_{\cdot 02} = 7{\cdot}824$).

Since this is only one among 30 (not completely independent) sub-experiments we could, of course, expect the figures for one or more of these sub-experiments to show what would have been by itself a probability of 1 in 20. At the beginning of the experiment, however, we laid down the hypothesis that we had no reason to expect significant results from one tumour type or characteristic more than another. It follows that there is, so far as our results *per se* are concerned, no reason to regard this as indicating a real correlation between age and increasing epidermoid differentiation in bronchogenic carcinoma. It is notable, however, that in our reading of the literature the tumour in which the correlation between increasing differentiation and increasing age has been most strikingly demonstrated (Geschickter and Denison, 1934) is also bronchogenic carcinoma. Consequently our figures, in so far as they are confirmatory, are also suggestive.

We did, in fact, although we have not given the table, estimate, using the method of analysis of variance, the probability with which the figures for epidermoid differentiation and age could have occurred by chance for epidermoid differentiation of (*a*) bronchogenic carcinoma, (*b*) carcinoma of the cervix, (*c*) carcinoma of lip. The probabilities were, respectively, c. 0·01; not significant; c. 0·05. In carcinoma of the lip and in carcinoma of the lung the sense of the association, although in each case "significant," was unfortunately opposite, i.e. as judged by each set of figures taken separately, carcinomas of the lung tend to be more differentiated at higher ages, and carcinomas of the lip less differentiated at higher ages.

We can summarize our findings thus far in the simple statement that there is no good evidence of a correlation between the age of the patient and the degree of any of the histological factors we have estimated, with the possible exception that carcinomas of the lung tend to be undifferentiated in younger patients.

In order to investigate this matter further we made another selection of slides from the files, all examples of bronchogenic carcinoma, all chosen and examined in the same way, using the same criteria. These slides did not include any of

those already used. They comprised 216 sections, 54 sections in each of the age groups 31–40, 41–50, 51–60, and 61 and over. Of the 54 patients in each group, 46 were male and 8 female.

The results are given in Table II. They were analysed by estimating the significance of the slope of the regression line of increasing epidermoid differentiation on increasing age.

TABLE II.—*Distinguishing Degree of Epidermoid Differentiation as Found in 216 Carcinomas of the Lung, 54 in Each of Four Age Groups—31–40 years, 41–50 years, 51–60 years and 61 years and over—giving the Number of Cases in Each Grade in Each Age Group.*

	31–40.	41–50.	51–60.	61 and over.
Grade I	40	36	33	33
Grade II	6	13	8	12
Grade III	4	4	10	8
Grade IV	4	1	3	1

The regression line was found by using the formula—

$$y - \bar{y} = b(x - \bar{x})$$

where y is the grade of epidermoid differentiation found, x is the age in years, and $b = \dfrac{\Sigma(y - \bar{y})\,(x - \bar{x})}{\Sigma(x - \bar{x})^2}$.

b, the slope of the best fitting regression line, was found to be 0·0052. The variance of b, using the formula—

$$V_b = \frac{1}{(n-2)\,\Sigma(x - \bar{x})^2}\left[\Sigma(y^2) - \frac{(\Sigma y)^2}{n} - \frac{[\Sigma y(x - \bar{x})]^2}{\Sigma(x - \bar{x})^2}\right]$$

was found to be 0·000028139. This means that figures which give a regression line with this small slope might easily have occurred by chance, and there is no significant correlation between increasing age and increasing degree of epidermoid differentiation shown by these figures.

It is to be noted, however, that the range of age was small since there were no more sections from patients aged 30 years or less available for study, and it might well be that it is the patients under 30 who give the curve its significance.

In summary we would say that there is a case for the existence of such a correlation though it cannot be very strong.

DISCUSSION.

Opinions in the literature on the subject are contradictory. Certainly there is no consensus of opinion that the malignancy of cancer as estimated clinically is greater in younger patients than in older patients. As far as we can estimate there is certainly no difference histologically. How far histological appearances are themselves correlated with growth rate and malignant behaviour in a carcinoma is, of course, a debatable matter, but in so far as they are an indication of the degree of clinical malignancy the evidence derived from them does not confirm the belief that tumours are more malignant at young ages.

We think that the data we have given add up to the statement that carcinoma at younger ages is not more malignant than at older ages, or at least that the contrary has not been proved.

SUMMARY.

The histological appearances of carcinomas from 105 patients aged 30 years or less have been compared with those of a similar number of patients in age groups 46–55 (41–50 for lung) and 70 and over (61 and over for lung). No significant differences have been found except doubtfully in carcinoma of the lung.

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SPONTANEOUS HEPATOCELLULAR AND CHOLANGIOCELLULAR CARCINOMA IN THE DUCK. AN EXPERIMENTAL STUDY.

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In July, 1944, during the course of a long-term investigation into avian neoplastic disease, a post-mortem examination of a duck showed it to have multiple liver tumours. Unfortunately, this case was not seen personally and no record was kept of any details apart from the sex of the subject—a female. However, material was taken for histological examination and a diagnosis was later made of liver-cell carcinoma. Primary cancer of the liver is known to occur in chickens, but is relatively uncommon. A careful search of the literature failed to bring to light any previous mention of this condition in ducks.

During the following month another case was found during a post-mortem on a White Campbell duck. In a preliminary note (Campbell, 1946) this duck was erroneously called an Aylesbury. Later that month a third case was observed in a Khaki Campbell duck. Both of these birds were in their second year and in all subsequent cases examined the ages of the birds varied between 2 and 3 years. So far, the condition was found to be confined to the liver, with no metastases visible to the naked eye.

At the time of writing, a total of 21 cases have been examined either as post-mortem subjects or in the terminal stages of the condition, while several cases have been utilized for experimental work. All cases occurred in the female subject and in the Campbell strain, either White or Khaki.

Epizootiology and symptoms.

It will be seen from Table I that out of the 22 ducks examined, 17 came from 4 flocks, designated Flock A, B, C and D. None of these was a large flock ; 15 ducks being the maximum number maintained. Two of these sources provided 3 cases each, a third provided 4 cases (one of which was not seen personally), and a fourth flock provided 8 cases from a flock of 12 ducks. One of the latter cases (Case 17) had 2 different simultaneous neoplastic conditions, namely a large thoracic histiocytic-sarcoma of undetermined origin, and a much smaller hepatoma.

Prior to the first observed case in 1944 a total of 21 ducks had been examined in 4 years, and no case of neoplastic disease was encountered. An examination of the records kept prior to 1939 showed that no case of liver carcinoma, or indeed of tumours of any sort, was observed in ducks. Since 1944, however,

in a total of 76 ducks examined, 22 had liver carcinoma, or an incidence relative to disease in ducks as a whole of nearly 29 per cent.

This duplication of cases in several small flocks suggested that an attempt should be made to trace the original source of the ducks composing these flocks. When this was done, the interesting fact came to light that in 2 instances (Flocks B and D) the ducks were obtained from an identical source in Cumberland. No information was obtained regarding the 2 remaining flocks.

Several birds suffering from this condition have been examined alive, and in the later stages of the disease the symptoms are very characteristic.

There is usually a history of loss of appetite and of lethargy. The duck shows a tendency to walk or stand in a more upright position than normal, thus simulating the gait and stance of a penguin. The abdomen becomes enlarged, although the bird tends to lose weight. There is frequently a history of greenish fluid evacuations. As the disease progresses, general weakness brings about the loss of locomotory power, and death supervenes either suddenly from an internal haemorrhage, or slowly from cachexia and exhaustion.

If examined during this late stage, a dropsical condition of the abdomen is apparent, and careful palpation shows the presence of an enlarged nodular liver projecting well beyond the caudal extremity of the sternum on the floor of the abdomen. There may be signs of respiratory embarrassment, due to increased abdominal pressure, or to lung involvement through metastatic spread, or to both these factors. Tapping the abdomen with a large-bore hypodermic needle results in the drainage of a considerable volume of clear green transudate with specific gravity of about 1·014. This often gives temporary relief, and life may be prolonged for a week or two.

Post-mortem findings.

The duck is frequently underweight, sometimes emaciated. On opening the abdomen a variable amount of fluid is found, usually clear yellow or green in colour. The liver is always enlarged, sometimes to an enormous degree, e.g. Case 7 (Table I), where it was nearly half the weight of the bird. Usually one lobe is mainly involved, but there is no definite predilection for right or left lobe. In a typical case, the liver tumours are nodular and bright green in colour and covered with a tense glistening membrane containing ramifying vessels.

Other tumours may take the form of firm, cream-coloured nodules, probably representing more recent growths not yet stained with bile. Haemorrhagic or necrotic nodules are not uncommon. In the author's collection of cases, the liver weight varied between 90 g. and 625 g., the weight of a normal liver being in the region of 75 g.

When the liver is incised, soft, sometimes pasty greenish tumours are found involving the whole organ. Each nodule is discrete, and separated from adjacent nodules by a thin zone of pale tissue, thus giving a trabecular appearance to the cut surface. These trabeculae may merge towards the centre of the tumourous lobe to form a broad, well-defined " trunk " from which the trabeculae radiate outwards like branches (Fig. 1).

The most common sites for secondary growths are lungs, kidneys and ovary. Other sites have been observed, e.g. intestine, oviduct and spleen. A number of cases showed no metastatic spread evident to the naked eye. When metastases

are obvious, however, they are characterized by a greenish-brown discrete type of growth. The lung is frequently riddled with such tumours, and they may attain a comparatively large size in the ovary. In the kidney they form spherical tumours projecting from the surface of the organ (Fig. 2).

Histopathology.

The structure of these primary liver tumours in the duck varies from the well differentiated adenomatous type of tumour in which metastasis is uncommon, to a frankly anaplastic type which is highly malignant. As no useful purpose could be achieved by attempting to describe all the intermediate grades, only the adenomatous and anaplastic forms will be described.

The adenomatous type of hepatocellular carcinoma bears a strong resemblance to embryonic liver tissue, being composed of anastomosing tortuous tubules or solid cords (Fig. 3). The liver cells forming these structures are cuboidal, columnar, or polyhydral with a faintly granular eosinophilic cytoplasm ; they contain a rather small, well defined nucleus, 3–4 μ. in diameter, and 1 or 2 small eosinophilic nucleoli, 1–2 μ. in diameter (Fig. 4). The walls of the tubules may be only one cell thick, or there may be several layers. Occasionally, broad sheets of cells form a trabecular pattern. The nuclei of those cells forming tubules are often situated centrally or adjacent to the lumen.

Mitotic division is not often seen. There may be attempts at the formation of a central vein and the cells immediately apposed to the endothelium are usually columnar in form. Connective tissue divides the tumour into irregular lobules and contains numerous aberrant bile ducts.

At the other extreme is the anaplastic type, which is characterized by an almost complete loss of differentiation towards a glandular structure. The cells are large and polymorphic with vesicular nuclei varying greatly in size and shape, some exhibiting hyper-chromatism and containing 1 to as many as 10 prominent eosinophilic nucleoli. Typical measurements for such cells are, nucleus 13·6 μ. and nucleolus 6·4 μ. The nuclear chromatin is often distributed as basophilic granules just within the nuclear membrane (Fig. 5). Both mitotic and amitotic divisions are common, with occasional atypical figures.

The intercellular reticulum is scanty and tends to enclose groups of cells. Occasional connective tissue trabeculae occur, containing capillaries, and wide blood vessels composed only of endothelium are scattered throughout the tumour. Emboli of tumour cells are frequently encountered in such vessels. Blood sinuses are frequent, with the blood in direct contact with the liver cells forming the cavities. Growth of the tumour appears to be mainly expansive.

In several instances a concomitant neoplasia of the bile ducts was apparent. The behaviour of such tumours upon metastasis is described below.

Bile duct or cholangiocellular carcinoma.

Typically, these tumours offer no diagnostic difficulties upon histological examination, owing to the close resemblance between the neoplastic cells and those composing normal bile ducts (Fig. 6). In some cases, however, anaplasia renders a differential diagnosis extremely difficult.

The cells composing these tumours have a slightly basophilic cytoplasm, with oval, vesicular nuclei possessing a delicate nuclear membrane and containing

1 or 2 small nucleoli. They show a tendency to form duct-like structures, which are normally detectable on careful search, even in the anaplastic type. The cells are frequently columnar and in such cases the nuclei are usually situated proximally, or remote from the lumen. Mitotic figures are common and the tumour appears to grow mainly by infiltration. The types of tumours encountered were as follows: 17 hepatocellular, 1 cholangiocellular, and 4 mixed.

Metastatic spread.

Spread by metastasis occurred in 10 cases out of the 22 examined. The findings are summarized in Table I.

TABLE I.—*Liver Carcinoma of the Duck.*

Case No.	Breed.	Sex.	Weight (kg.).	Type of tumour.	Weight of liver (gm.).	Metastases.
1	?	F.	..	H.C.	..	..
2	White Campbell	F.	1·8	„	185	..
3	Khaki „	F.	1·6	„	..	..
4	White „	F.	2·5	„	463	..
5	Khaki „	F.	1·6	„	165	..
6	„ „	F.	2·5	„	..	Ovary and intestine.
7	„ „	F.	1·3	„	625	..
8	„ „	F.	2·0	„	..	..
9	„ „	F.	1·1	Mixed	245	Ovary and kidneys.
10	?	F.	2·7	„	..	Ovary.
11	Khaki Campbell	F.	1·1	H.C.	..	..
12*	„ „	F.	1·8	„	210	Ovary.
13*	„ „	F.	1·1	„	325	..
14	„ „	F.	1·8	„	135	..
15	„ „	F.	1·6	C.C.	160	Lungs and kidney.
16*	„ „	F.	1·3	H.C.	260	..
17	„ „	F.	1·5	„	90	..
18*	„ „	F.	1·6	Mixed	180	Lungs.
19	„ „	F.	1·6	H.C.	412	„ kidney and ovary.
20*	„ „	F.	1·8	Mixed	330	„ and ovary.
21	„ „	F.	2·0	H.C.	455	„ „ „
22	„ „	F.	1·6	„	280	„ ovary, kidney and spleen.

* Used for experiments.
Normal weight of liver c. 75 g.
„ „ adult Khaki Campbell duck 2·25 kg.
Of the above, Flock "A" contributed 3 cases (3, 5, 8).
„ "B" „ 3 „ (9, 12, 15).
„ "C" „ 3 „ (6, 11, 13).
„ "D" „ 7 „ (4, 7, 16, 17, 18, 19, 20).
N.B.—Another case was seen by a second observer, in Flock "B."
Key.—H.C., Hepatocellular carcinoma.
C.C., Cholangiocellular carcinoma.
Mixed, Both types present in liver tumour.

It will be seen that the tumour most commonly metastasizes to the lungs (Fig. 7) and ovary. Metastasis occurs mainly by blood stream (Fig. 8) for tumour emboli are frequently seen within small veins.

In the case of mixed tumour of the liver, where both hepatocellular and cholangiocellular carcinoma are present, it will be noticed that only one type of neoplastic element metastasizes. Only 4 such mixed tumours were found in which metastasis had occurred. In 3 of these cases the secondaries were of the bile duct type, while in the remaining case it was the liver cell moiety which had metastasized.

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In Table I the types of tumour are noted. In 22 cases, in a total of 17 hepatocellular tumours, 5 showed metastatic spread, the 1 cholangiocellular tumour had metastasized and of the 4 mixed tumours, as mentioned above, all had metastasized.

RESULTS OF CULTIVATION, SUBCULTIVATION AND TRANSMISSION EXPERIMENTS.

(1) *Intravitelline inoculation.*

Preliminary experiments 1 and 2 included the injection of tumour cells into the yolk-sac of 24 fertile eggs, and a similar experiment with cell-free material. The results were not encouraging. In Experiment 2, for example, out of 5 eggs injected with a filtrate of the tumour, 2 embryos died and the rest survived to hatch, and of the 3 controls injected with sterile normal saline, 1 died and the rest hatched normally. However, the 2 dead embryos from the egg injected with filtrate showed numerous skin petechiae, and similar lesions on the chorio-allantois, whilst the allantoic fluid was a clear red colour. It was bacteriologically sterile. This haemorrhagic condition was not seen in the dead control embryo. The red allantoic fluid from one of the dead embryos was injected into the yolk-sacs of six 7-day-old embryos, 4 of which died within 48 hours, a 5th died 4 days after injection whilst the last survived and hatched normally.

It was thought that these findings might indicate the presence of some specific lethal factor in the filtrate, but in view of previous unfavourable results obtained with a number of different tumours injected into the yolk-sac, and of negative results when duck liver tumour cells and filtrate were injected, the method was discarded in favour of cultivation on the chorio-allantoic membrane.

(2) *Cultivation on the chorioallantoic membrane.*

Experiment 3.—The technique used in this and in all the subsequent egg cultivation experiments detailed was essentially that of Beveridge and Burnet (1946).

Eight 11-day incubated eggs were drilled, and a small fragment of liver carcinoma from case number 16, implanted on the chorioallantois on the following day. Broths were inoculated at the same time with tumour fragments and were later seen to be sterile. On the 18th day of incubation when the implants had been in position for 6 days, 6 eggs were opened and 3 showed a distinct greenish discolouration of the underlying shell membrane. Below this, small green tumours were growing on the chorioallantois, the largest measuring 4 × 3 × 2 mm. These growths were vascularized by blood vessels from the membrane and were surrounded by a somewhat opaque thickened zone (Fig. 9 and 10). One of the 2 remaining eggs was allowed to hatch and produced a healthy chick. Examination of the membrane immediately after hatching showed that it bore a small tumour. The 8th egg was opened on the 19th day, and also contained a tumour which was implanted into the anterior chamber of a guinea-pig's eye.

In this experiment, therefore, 5 out of 8 eggs contained small tumours.

Experiment 4.—Five 13-day embryonated eggs were inoculated each with 0·02 ml. 24 hours old filtrate prepared by grinding up liver tumour tissue in a glass homogenizer, centrifuging, and filtering, using a Seitz type filter and Ford's " sterimat " grade G.S. The filtrate was inoculated on to the chorioallantois in

the usual way, and the eggs sealed with paraffin wax and returned to the incubator.

They were opened on the 5th day after inoculation. Slight opacities were noticed in the membranes, which were not quite so flexuous as normal. Subsequent histological examination, however, showed little abnormality apart from some slight ectodermal proliferation.

Experiment 5.—Ten 5- to 6-day-old fertile eggs were implanted on 18.iv.47 with small fragments of liver carcinoma from Case 18. A liver fragment and some of the saline in which it was minced, were placed into broth which on the following day was found to be sterile.

On the 20th of the month 2 embryos were found to be dead. One showed an implanted fragment adhering to the membrane, which was taken for histological examination.

Two further embryos were dead on the 23rd. Both had tumours growing on the membrane. One egg with a living embryo was also opened but there was no sign of a tumour.

The remaining eggs were opened on the 30th. Of the 5, 4 had small growths in the membrane—disappointingly small considering the cultivation period (12 days). Three of these were removed, dissected away from the membranes and reimplanted into two 7-day-old embryonated eggs. The remaining 1 was taken for histological examination. On 10.v.47 the 2 eggs were opened and 1 contained a small dark green growth 2 × 2 × 2 mm. in size. Further subcultivation was not attempted.

Experiment 6.—A biopsy was performed on a duck with liver carcinoma (Case 20). The tumour tissue was crushed off the margin of the liver using an emasculator for caponizing cockerels. Haemorrhage was controlled with saline packs and adrenaline. Fragments of tissue were immediately implanted into 5 eggs, 4 containing 10½-day-old embryos, and 1 being 9 days.

The eggs were opened 9 days later; 4 contained tumours. The largest of these (6 mm. diameter) was divided into 4, and reimplanted into 4 eggs of 8 days' incubation. The remainder was taken for histological study. The eggs containing the second passage implants were opened 10 days later, and 3 contained small tumours between 2–3 mm. diameter.

Later a further biopsy on Case 20 provided material to implant into three 8-day embryonated eggs. The duck died near the termination of the operation owing to too deep anaesthesia. The eggs were examined 10 days later, and 2 contained small tumours, about 3 mm. diameter, surrounded as usual by an opaque area.

(3) *Cultivation in the anterior chamber of the eye.*

Experiment 7.—Using the technique described by Greene (1941), small tumour fragments from a duck liver (Case 16) were introduced into the anterior chamber of a guinea-pig's eye. Fourteen days later, the eye was examined with the aid of an ophthalmoscope and 2 small brownish-green growths were observed, 1 on the upper pupillary border, and 1 laterally placed. The animal was destroyed on the 18th day of implantation and one of the growths measuring 4 × 3 × 2 mm. removed and implanted into three 9-day embryonated eggs, which upon examination on the 18th day of inoculation showed no tumours on the membrane. The eye was prepared for histological examination of the remaining tumour.

Experiment 8.—A small liver carcinoma grown for 7 days on the chorio-allantois was implanted into the anterior chamber of the eye of a guinea-pig. It failed to grow and was eventually absorbed.

Experiment 9.—This was a repeat of Experiment 7. A fragment of liver tumour obtained at biopsy (Case 20) was introduced into the anterior chamber as before. The implant failed to grow and was rapidly absorbed.

(4) *Direct transmission experiments (homologous).*

Experiment 10.—A Khaki Campbell duck, 2 months old, had its liver exposed by intercostal incision, and was given an intra-hepatic injection of 1·0 ml. suspension of liver carcinoma in saline. The original tumour was obtained from Case 13. Sixteen months later the duck was killed and found to have typical liver tumours, which proved to be hepatocellular carcinoma. There were no metastases.

Experiment 11.—A 5-month-old Khaki Campbell duck was given an intra-hepatic injection of 1·0 ml. suspension prepared from a liver carcinoma obtained from Case 16. The material used was not more than 1½ hours old. The duck was killed at the end of 16 months, and the liver contained numerous tumours which histologically were cholangiocellular carcinomas. Again there were no metastases.

Experiment 12.—Representative portions of liver carcinoma from Case 18 were injected as a suspension in normal saline into the following Aylesbury ducklings (it was not possible to get Khaki Campbell ducklings at short notice):

(1) Four 3-day-old ducklings received 1·0 ml. into the peritoneal cavity.

(2) Four received 0·5 ml. intravenously.

(3) Four received 0·5 ml. intramuscularly. (Actually the injection was practically subcutaneous, the pectoral muscles being poorly developed at this age.). This latter group also received 1·0 ml. cell-free filtrate prepared from the tumour into the peritoneal cavity.

One duckling in group (1) died the following day. Two were destroyed 2 months later and showed no lesions upon post-mortem examination.

In group (2) 2 were destroyed 2 months later and showed no lesions.

In group (3) and in the remainder of groups (1) and (2) the ducks were destroyed 14 months later and upon post-mortem examination showed no abnormality.

Experiment 13.—Fragments of liver tumour obtained at biopsy from Case 20 were implanted by means of a wide bore hypodermic needle into the liver of another Khaki Campbell duck, exposed as before by intercostal incision. The bird was then 8 months old. Fourteen weeks later it was observed to be weak, losing weight, and to have an enlarged abdomen. It was destroyed and examination showed a large liver tumour weighing 280 g. with metastases in the lungs, kidney and ovary.

Histological examination of this tumour showed it to be a hepatocellular carcinoma. The original tumour (Table II) was a mixed type, but with cholangiocellular metastases.

TABLE II.—*Metastatic Spread of the Duck Liver Carcinoma.*

Case No.	Nature of primary tumour.	Organs involved.	Nature of metastasis.
6	Liver cell carcinoma	Intestine and ovary	Liver cell carcinoma.
9	Mixed (liver and bile duct)	Ovary and kidney	,, ,,
10	Mixed	Ovary	Bile duct ,,
12	Liver cell carcinoma	,,	Liver cell ,,
15	Bile duct ,,	Lungs and kidney	Bile duct ,,
18	Mixed	Lungs	,, ,,
19	Liver cell carcinoma	Lungs, ovary and kidney	Liver cell ,,
20	Mixed	Lungs and ovary	Bile duct ,,
21	Liver cell carcinoma	,, ,,	Liver cell ,,
22	,, ,,	Lungs, ovary, kidney and spleen	,, ,,

(5) *Direct transmission experiment (heterologous).*

In view of the transmissibility of at least 1 tumour from 1 species of bird to another, i.e. the Fujinami sarcoma of fowls, which has been transmitted to ducks, it was considered worthwhile attempting a similar experiment with the duck liver carcinoma.

Experiment 14.—Three 6-week-old Brown Leghorn chickens each received 1·0 ml. tumour suspension into the peritoneal cavity, and 1·0 ml. into the left pectoral muscle. The original tumour was from duck Case 18.

On the 3rd day 1 chicken was found in a dying condition and was killed. The pectoral muscle contained a small greenish-brown growth which was taken for section. This was approximately 66 hours subsequent to injection. No detectable growth was found in the peritoneal cavity.

A 2nd chicken was found dead, 6 days subsequent to injection. There were small fusiform tumours in the pectoral muscle and in the peritoneum.

A 3rd chicken survived and was destroyed 2 months later. No sign of tumour growth was found.

Histology of cultivated and transplanted tumours.

The structure of the implants on the chorioallantoic membrane is typically that of the original carcinoma (Fig. 11). There seems to be no tendency to revert to a more undifferentiated type. The liver-cell tumour forms solid cylinders each surrounded by ramifying reticulum fibrils (Fig. 12). The tumour is well supplied with blood vessels derived from the chorioallantois. Mitotic division is frequently seen, but some areas, probably those not vascularized in time, are undergoing necrobiosis, the nuclei showing pyknosis, karyorrhexis and karyolysis. Such areas are infiltrated with phagocytic cells such as macrophages. The capsule is composed of fibroblasts derived from the mesenchymal tissue of the chorioallantoic membrane. It is usually infiltrated by pseudo-eosinophils, and may contain epithelial elements resembling bile ducts.

When a portion of such a tumour is transplanted a second time, the resultant growth is usually small and contains considerable necrotic areas. In those that survive, however, the same structure is apparent, but the bile duct elements, if present, appear to persist longer than the liver cell moiety.

The bile duct type (Fig. 13) is characterized by epithelial cells arranged in the form of ducts or villiform structures, which may take on a cystic appearance.

It has already been shown that the original liver tumour in the duck often varies a great deal in structure in different parts. It is obvious, therefore, that

the cultivated tumours will also vary according to the site from which they were taken. This possibly accounts for the differences between the original tumour and the cultivated or transplanted tumours shown in Table III.

TABLE III.—*Nature of Cultivated and Transplanted Duck Liver Carcinoma.*

Case No., Exp. No.	Diagnosis of original tumour.	Diagnosis of cultivated tumour (C.A. membrane).	Diagnosis of subcultivated tumour (C.A. membrane).	Diagnosis of transplanted tumour (homoplastic and heteroplastic).
16 (1, 5)	L.C. carcinoma	L.C. carcinoma	..	L.C. carcinoma
18 (3, 11)	Mixed type	B.D. ,,	Necrotic	Mixed type
20 (4, 10)	,, ,,	(*a*) L.C. carcinoma	L.C. carcinoma	..
	,, ,,	(*b*) Mainly L.C. but few bile ducts	..	L.C. carcinoma

Key: L.C. = Liver cell.
B.D. = Bile duct.
Mixed = Both liver and bile duct elements present.

Histology of chorioallantoic lesions.

In every instance where a liver tumour was successfully cultivated on the chorioallantois, a zone of opacity of varying extent developed in the membrane adjacent to the tumour (Fig. 9). This area was only obvious when the membrane and growth were examined against a dark background. Fig. 10 shows the same tumour-bearing membrane photographed against a white background, and the lesions are then barely perceptible.

Upon histological examination, an interesting picture is revealed. The membrane adjacent to the tumour is thickened and very vascular. The thickening is due in the first place to a pronounced proliferation of the ectodermal cells, which become grossly hypertrophied and vacuolated. They measure from 15–30 μ in diameter (Fig. 14). The nuclei of these cells are irregular and densely staining. Fine cytoplasmic fibrils occupy the cell body, which has a well-defined outer membrane. Eosinophilic masses occur in some cells, resembling to some extent the inclusion bodies in a fowl-pox lesion of the chorioallantois.

Secondly, there is a proliferation of connective tissue in the membrane. Lying enclosed in this stroma are epithelial structures resembling the "cell nests" of squamous-cell carcinoma. These are eosinophilic concentric bodies whose outer coats are composed of flattened epithelial cells derived from a down-growth of the chorioallantoic ectoderm. Passing towards the centre of the structure, these cells become hyalinized and lose their nuclei by fragmentation. The centres of the bodies are composed of a mass of dead, occasionally vacuolated cells (Fig. 15, 16).

Table IV shows that tumour implants stimulated ectodermal proliferation to

TABLE IV.

Material.	Virus.	Experiment No.	Reaction of adjacent membrane.	
			Ectodermal proliferation.	"Cell nest" formation.
Duck liver carcinoma	?	3	+	+
Filtrate	,,	4	+	−
Duck liver carcinoma	,,	5	+	+
,, ,,	,,	5 (subcultivation)	+	+
,, ,,	,,	6	+	+
,, ,,	,,	6 (subcultivation)	+	+
,, ,,	,,	6 (2nd biopsy)	+	+

the extent of " cell nest " formation. Filtrate on the other hand, simply caused ectodermal proliferation.

Histology of transplanted tumours (heteroplastic grafts).

In Experiment 14, duck liver carcinoma suspension was injected by various routes into young Brown Leghorn chickens, where the tumour grew successfully up to 6 days.

The nodule removed from the pectoral muscle after 66 hours shows groups of viable carcinoma cells, forming distinct glandular acini. Some cells are actively dividing, and the graft appears to be infiltrating the muscle (Fig. 17). The tumour tissue is vascularized and in places a connective tissue stroma contains epithelial structures resembling bile ducts. There is some necrosis of the muscle adjacent to the graft, probably due to trauma following injection, and an early host reaction around the foreign cells is indicated by the presence of infiltrating histiocytes, macrophages, pseudo-eosinophils and a few lymphocytes.

In the peritoneum, the host reaction appears to be much more vigorous. The nodule shows a dense connective tissue base (thickened peritoneum), growing from which is a mass of vascularized lymphoid tissue containing a few islands of surviving liver cells (Fig. 18).

The 6-day graft (Fig. 19) shows a bile duct type of tumour, with a villiform structure. It is still actively growing and infiltrating the muscle, but there are only a few surviving liver cells in the form of acini, and these are engulfed in a massive lymphocytic infiltration.

In Experiment 7, fragments of liver carcinoma (Case 16, liver cell type) were implanted into the anterior chamber of the eye of a guinea-pig. Two tumours grew. On the 18th day the animal was destroyed and the eye opened. One tumour measuring $4 \times 3 \times 2$ mm. was used for further transplantation experiments and the other smaller growth was left *in situ*, and the eye prepared for histological examination.

This shows a small tumour composed mainly of an undifferentiated mass of epithelial cells attached to the iris (Fig. 20). Some attempt to form cord-like structures may be seen. The growth is surrounded by a zone of fibroblasts and is in parts infiltrated with lymphoid cells. Vascular connections with the capillaries of the iris can be seen.

DISCUSSION.

In a previous communication, Campbell (1949), reported the results obtained by implanting various spontaneous and chemically induced chicken and mammalian tumours into the fertile hen egg. Two main sites were used, namely the yolk-sac and the chorioallantoic membrane. Yolk-sac cultivation did not give satisfactory results, this also being the experience of Twombly and Meisel (1946); although Taylor, Thacker and Pennington (1942), and several other workers claimed a high perentage of intravitelline growths with the particular tumours used. These were mainly mouse and rat mammary carcinomata, and the poor results obtained by others, using different tumours, seems to indicate that the environment provided by the chick embryo yolk-sac is peculiarly suitable for the metabolic requirements of mammary cancer cells.

On the other hand, most of the tumours implanted on the chorioallantois

grew successfully and were readily visible, and the death rate of embryos was considerably lower than after yolk-sac injection. The sudden occurrence of a number of cases of hitherto unrecorded primary carcinoma of the duck's liver provided material for cultivation by various methods, with the main object of searching for a transmissible factor or virus. The chorioallantoic method of cultivation, therefore, seemed particularly suitable, and was extensively used.

There appear to be two possible explanations of the etiology of this condition. Firstly, the disease might be associated with a transmissible factor. It is known that the mouse mammary cancer factor of Bittner is transmitted from mother to offspring by the milk, and this is one of the very few known instances of a tumour of epithelial nature associated with a virus-like principle. It was felt that the duck liver carcinoma might also contain a factor transmissible probably *via* the egg, which might be capable of propagation in the same manner as the milk factor of mice.

Experiments designed to test this theory, however, have given negative results as far as transmission by cell-free material is concerned. The only evidence for an associated virus has been that obtained from the cultivation of the tumour on the chorioallantoic membrane, where in every instance typical lesions developed in the ectoderm adjacent to the implant, or subsequent to inoculation with filtrates of the tumour. Also, yolk-sac injections resulted in

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EXPLANATION OF PLATES.

FIG. 1.—Liver, and cut surface of one lobe, showing a hepatocellular carcinoma of a Khaki Campbell duck. 4/5th natural size.

FIG. 2.—Metastases of hepatocellular carcinoma in the lungs, ovary and kidneys of a Khaki Campbell duck. 4/5th natural size.

FIG. 3.—Hepatocellular carcinoma. Khaki Campbell duck. H. & E. × 145.

FIG. 4.—Detail of duck hepatocellular carcinoma. H. & E. × 660.

FIG. 5.—Anaplastic type of duck hepatocellular carcinoma. H. & E. × 690.

FIG. 6.—Cholangiocellular carcinoma. White Campbell duck. H. & E. × 145.

FIG. 7.—Metastasis of bile duct moiety of a primary mixed tumour to the lung of a Khaki Campbell duck. H. & E. × 56.

FIG. 8.—Embolus of liver carcinoma cells within vein of intestinal wall, Khaki Campbell duck. H. & E. × 660.

FIG. 9.—Duck liver carcinoma cultivated on the chorioallantoic membrane. Note surrounding zone of reaction. × 2.

FIG. 10.—The same, photographed against a white background. The reaction zone is not now apparent.

FIG. 11.—Structure of duck hepatocellular carcinoma growing on the chorioallantois. H & E. × 56.

FIG. 12.—Detail of duck hepatocellular carcinoma growing on the chorioallantois showing reticulum. Wilder's method. × 260.

FIG. 13.—Cholangiocellular carcinoma of the duck, growing on the chorioallantois. The tendency to form duct-like structures is well shown. H. & E. × 56.

FIG 14.—"Ballooned" ectodermal cells of the chorioallantois, adjacent to an implanted duck liver carcinoma. H. & E. × 530.

FIG. 15.—"Cell-nests" in the chorioallantois, adjacent to implanted liver carcinoma. Mallory's stain. × 56.

FIG. 16.—Detail of a typical "cell-nest" in the chorioallantois. Mallory. × 260.

FIG. 17.—Growing periphery of duck liver-cell carcinoma in pectoral muscle of a young chicken 66 hours after implanting. H. & E. × 260.

FIG. 18 —Lymphocytic reaction to the duck liver carcinoma, 6 days after implantation into the peritoneum of a chicken. H. & E. × 260.

FIG. 19.—Cholangiocellular carcinoma of the duck, infiltrating pectoral muscle of a chicken, 6 days subsequent to implantation. H. & E. × 56.

FIG. 20.—The duck liver-cell carcinoma growing in the anterior chamber of a guinea-pig's eye. A tendency to grow in cords is to be noted. H. & E. × 260.

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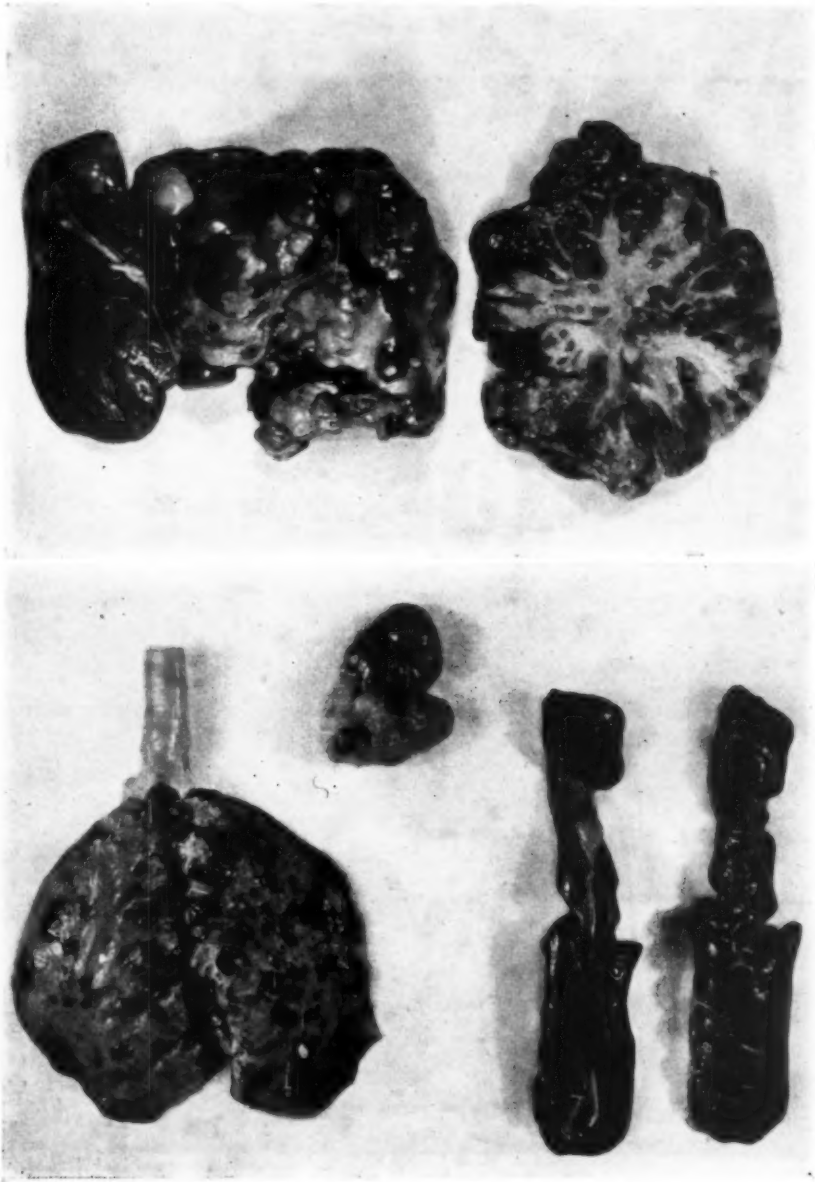

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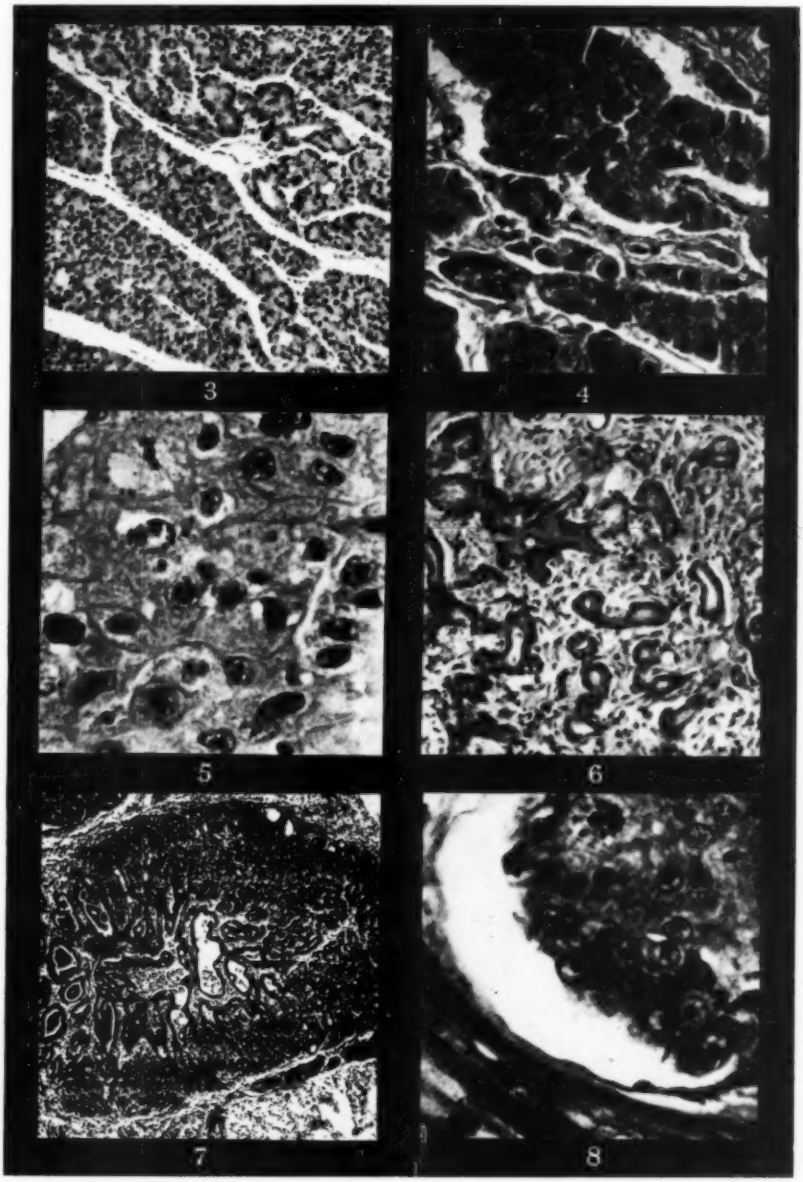

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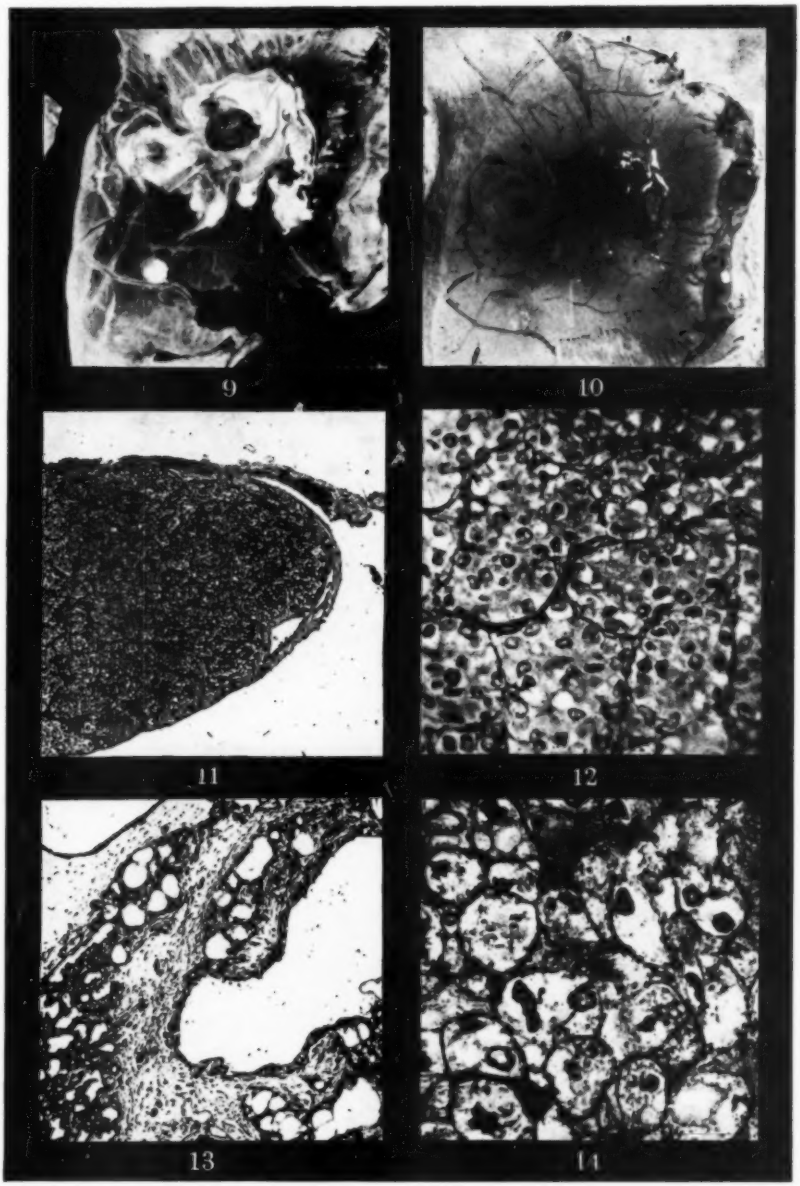

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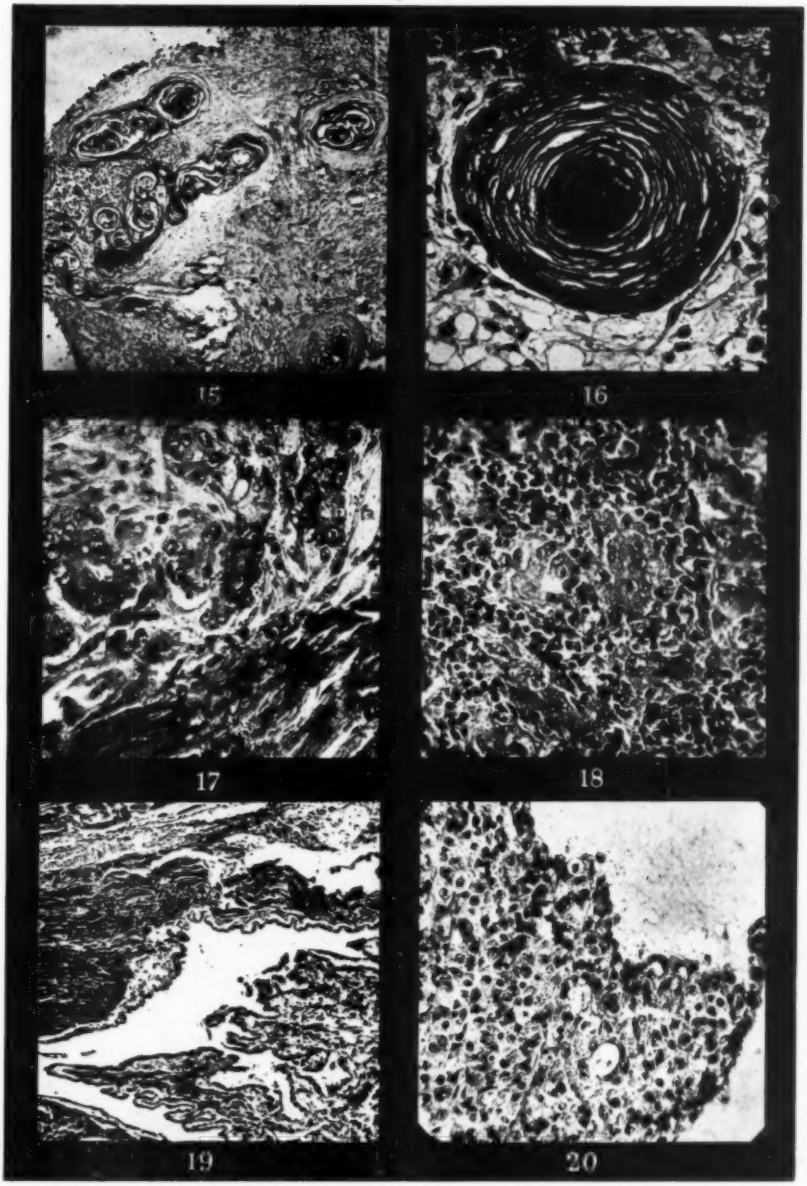

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the development of haemorrhages and death of the embryo, and the allantoic fluid from such embryos caused the death of embryos upon a second passage into eggs.

With regard to the ectodermal lesions, it has been shown that they reslut from the implantation of most tumours, also embryonic tissue, but not from normal adult tissues. It was suggested that these findings might indicate the presence of growth-stimulating substances in neoplastic and embryonic tissues, and supporting evidence for this hypothesis was produced (Campbell, 1949). It is clear, therefore, that the ectodermal lesions described cannot be regarded as evidence for the presence of a virus *per se*, although the lethal effect on embryos of injecting tumour material into the yolk-sac could conceivably be interpreted as such.

It is possible that if a virus is present in these tumours, it is not capable of demonstration by the methods used. It is well known that Rous I virus undergoes phases of inactivity when for a period it is difficult to induce tumours by filtrates. Similar inhibitors might be responsible for the failure to transmit the duck tumour by filtrate. The only successful transmissions were accomplished by the direct implantation of tumour tissue into the livers of recipients of the same breed. It is not surprising that the tumour could not be transmitted to young chickens, but the survival of these heterologous grafts for at least 6 days in the muscle and peritoneum of chickens is rather remarkable.

The second possible explanation of this apparent outbreak of liver cancer, is a genetic one. The condition was only encountered in a single breed of ducks, with the one exception where the bird was of a closely related strain. As far as could be ascertained the individuals in the small flocks involved bore either the sister or cousin relationship to each other, whilst the tracing of 2 separate affected flocks to a common source in the North of England may be a highly significant fact.

Turning to the spread of the tumour in the body, Cases 6 and 22 are of some interest owing to the sites of secondary deposits, respectively intestine and spleen. In the literature on primary liver cell carcinoma in the human subject, splenic metastases are mentioned but rarely. Cappell (1925) described a case of multiple microscopic metastases in the spleen in a case of liver cell carcinoma and remarks on the smallness of these secondaries in comparison with the large growths in the lungs, bronchi, pleura, heart and brain ; but he offers no explanation as to how the spleen becomes involved. There appear to be 2 possibilities, firstly, a retrograde spread along the hepatic vein to the coeliac vein, and thence back to the spleen, or, secondly, a complete circuit of the vascular system through the pulmonary circulation and so to the spleen *via* the splenic artery. In the one case encountered in the duck, pulmonary metastases occurred, and so this second hypothesis may be the correct one. Once the lungs are involved the tumours there may also metastasize ; tumour cells or emboli could then reach the spleen without much difficulty. A direct spread *via* the lungs seems unlikely in view of the formidable barrier offered by the pulmonary capillaries.

Fox and Bartels (1928) favour the view that in the human subject the spleen and mesenteric veins may be invaded by retrograde extension along the portal vein. In Case 6, besides serosal implantation on the wall of the intestine, tumour emboli were found within the veins of the gut wall, and their presence could be accounted for in this manner.

SUMMARY AND CONCLUSIONS.

A hitherto unrecorded neoplastic disease affecting the liver of the 2–3 year old female Khaki Campbell duck has been investigated, and details are given of the history, symptoms, post-mortem findings and pathology, together with experimental data resulting from cultivation by various *in vivo* methods.

It is concluded that while an associated transmissible factor cannot be definitely excluded, certain evidence has been collected that a genetic factor may probably be involved in its etiology.

I wish to express my gratitude to Professor A. Murray Drennan of the Pathology Department, University of Edinburgh, for his interest in this work, and for numerous suggestions as to the manner of development of the investigation.

All the fertile eggs were obtained from Dr. A. W. Greenwood's flock of Brown Leghorns, and I have pleasure in recording my appreciation of his co-operation in this respect, and for much help in other directions.

The histological preparations are the work of Messrs. Cockburn and MacKenzie.

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The colour photographs are the work of Mr. Richard Hood, Head Technician at the Royal (Dick) Veterinary College, Edinburgh.

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THE EFFECTS OF CASTRATION AND STILBOESTROL ON PROSTATIC TUMOURS IN MICE

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It is generally agreed that prostatic cancer does not develop spontaneously in rodents. Transplantable tumours of the rodent prostate have been induced following inoculation of the gland *in situ* with carcinogens, but the resulting growths have been either sarcomas or squamous-cell carcinomas (Moore and Melchionna, 1937; Dunning, Curtis and Segaloff, 1946; Horning and Dmochowski, 1947). Such kinds of prostatic cancer rarely occur, however, in man (Willis, 1948).

In view of the need for an experimental investigation into the effects of orchidectomy and endocrine therapy on the behaviour and growths of prostate tumours, new methods of tumour induction were undertaken in pure line mice, with the hope of obtaining a transplantable adeno-carcinoma. The successful induction of glandular carcinomas in mice arising from subcutaneous implants of adult prostatic epithelium impregnated with crystals of 20-methylcholanthrene has already been described in a preliminary communication (Horning, 1946)

The object of this paper is to trace in some detail the histogenesis of the induced prostatic carcinomas and to describe the influence of castration and sex hormone administration on their growth.

TECHNIQUE.

The method of tumour induction consists in isolating small strips of prostatic epithelium from either the anterior, ventral or dorsal lobes of the gland, and wrapping the pieces of epithelium around crystals of 20-methylcholanthrene prior to grafting the whole subcutaneously into host mice of the same sex, age and strain. The fragments of epithelium were isolated under a dissecting microscope with the aid of an iridectomy knife. The carcinogen was then placed in contact with the surface of the epithelium, and inserted into a Bashford transplanting needle, care being taken to shield the carcinogen from the connective tissues of the host animal. In some instances as many as three subcutaneous primary grafts were made on each side of the belly of a single host mouse to give a series of prostatic tumours growing under identical hormonal conditions. The technique is similar in some essentials to that previously employed by Greene (1945), and Rous and Smith (1945), except for the important difference that the present experiments involved tumour production from adult, not embryonic tissues.

The influence of bilateral orchidectomy upon the growth rates of glandular and squamous cell prostatic carcinomas was investigated in 6-months-old mice, which had previously been castrated before attaining puberty. The tumours

used in these experiments were in their twelfth passage of serial transplantation. Prostatic tumours undergoing regression, following transplantation into orchidectomized mice, were treated with testosterone propionate, each mouse receiving a dose of 1 mg. dissolved in oil every 24 hours.

Two types of prostatic carcinomas, an actively secreting glandular cell tumour and non-secreting squamous-cell tumour, both in their eighth generation of serial transplantation, were selected for determining the influence of stilboestrol on the behaviour of tumour growth. Each mouse in these groups received a 1 mg. pellet of diethylstilboestrol by subcutaneous implantation into its right flank according to the technique of Deanesly and Parkes (1937). The hormone pellets were inserted at the time of tumour transplantation, and the same dosage was repeated every 14 days.

The growth rates of all transplanted tumours were recorded weekly on silhouette charts.

All material for histological examination was fixed either in Zenker-formol or else in alcoholic Bouin, and subsequently stained with haematoxylin and eosin.

RESULTS.

Occurrence of Tumours.

Repeated experiments on tumour induction by this technique have demonstrated that neoplasms develop more rapidly, and arise in greater numbers, when prostatic epithelium is obtained from young mice in preference to old ones, provided the implants are grafted into host mice of the same sex, age and strain.

For instance, out of 25 male mice, old breeders over 10 months old, all of which received prostatic grafts impregnated with the carcinogen, only six developed palpable tumours after four months, whilst in a group of 25 young virgin males, 8 weeks' old, 19 developed tumours within eight to twelve weeks. For this reason prostatic tissue for purposes of grafting was always obtained from mice under twelve weeks of age.

The observations described in this communication were made on a total of 75 Strong A male mice of the above age. Mice in groups of 10 received grafts at intervals covering a period of 18 months. Small primary palpable tumours appeared from seven to twelve weeks after grafting. Out of a total of 75 treated mice, 54 developed tumours. Histological examination showed that 42 of these were glandular cell carcinomas. 10 were of the squamous variety, and 2 were spindle-cell sarcomas. Sixteen homologous grafts failed to grow, and were found at autopsy not to have become vascularized.

In order to determine the early phases of carcinogenesis within the primary grafts a further 30 mice received prostatic implants, and were sacrificed at intervals ranging from two weeks to five months after implantation. A control group of 35 mice received grafts of prostatic epithelium without the carcinogen, and were killed at intervals ranging from two weeks to six months after implantation.

Histogenesis of Tumours.

Before describing the early malignant changes which develop in prostatic grafts following impregnation with the carcinogen, it will be advisable at this stage to discuss briefly the probable homologies, and also the anatomical differences which exist between the human and rodent prostates.

The mouse gland, like that of the human, arises as a series of outgrowths from the embryonic urethra at its point of origin from the urinary bladder. The mouse gland consists of paired anterior, ventral and dorsal lobes, together with a small median gland, which may be absent or considerably modified in certain pure line mice. The human prostate, on the other hand, consists of two lateral lobes, which are practically fused together, with an anterior portion and an isthmus or middle lobe which, as in the rodent, surrounds the urethra. Unlike the human gland, the mouse prostate has no uterus masculinus or its equivalent, nor is it encapsulated. A difference exists also in the number of ducts which open independently into the urethra. Thus in the human gland there are as many as 32 ducts (Maximow and Bloom 1948), whilst in the mouse there are only six. A fibromuscular stroma consisting of dense connective tissue is common to both types of gland, but the mouse gland possesses a less elastic arrangement of smooth muscle fibres which in the human prostate surround the alveoli. The secretory epithelium is almost identical in its histology in the two species except for minor variations in the appearance of a basement membrane, which is better defined in the mouse. In both the human and rodent glands the secretory epithelium is thrown into folds which project into the glandular lumina, and though this folding varies considerably with the functional state of the gland, it is more pronounced in the rodent gland (Fig. 2 and 3). The cytoplasm of the glandular epithelium in both species contains numerous secretory granules, the majority of which are of lipids. Cytologically treated mouse material shows the secretion in both forms to consist of protein with fine lipoidal droplets in suspension. The alveolar concretions of the human prostate form characteristic structures, frequently becoming calcified, whereas the stored secretion of the mouse gland is usually evenly dispersed and lacks concretions. Hyperplasia of the prostate never occurs spontaneously in rodents, but a similar condition arises in mice following prolonged treatment with oestrogen, which induces a pronounced metaplasia of the prostatic epithelium in the dorsal and ventral lobes, involving, as in man, urethral obstruction, retention of urine and bilateral hydronephrosis (Fig. 1).

Control grafts implanted without the carcinogen.

Isolated fragments of prostatic epithelium without carcinogen continue to secrete prostatic fluid following successful implantation under the skin of host mice of the same sex and strain (Fig. 4). In such control grafts, fixed one and six months after implantation, distension of the alveoli occurs to a degree far in excess of what is typical of the normal gland *in situ*, and leads frequently to a condition of cystic dilatation (Fig. 4 and 2). The degree of the alveolar distension varies considerably with the age of the graft Fig. 4 shows a section through a control implant from the dorsal lobe, fixed after 12 weeks, in which all the alveoli are considerably distended. In cytological preparations there is some indication that the secretion is deficient in lipids in grafted alveoli as compared with those in the normal intact gland. In both control and experimental grafts there is evidence that the secretion is slowly dispersed and absorbed within the tissues of the host. It will be noted that the epithelium in control grafts is not hyperplastic, and hence it is important to distinguish as clearly as possible between growth in the grafts containing the carcinogen which is primarily due

to a failure in the release of the secretion, since the ducts are no longer patent, and proliferation which might be due to the direct action of the carcinogen.

Neoplastic changes in grafts implanted with the carcinogen.

The carcinogen, which is placed in direct contact with the living tissue without a solvent such as oil or lard, induces very little foreign body reaction, necrosis or residue within the graft which might mask or conceal the actual areas where neoplastic changes first arise. Thus it is possible to study serial sections of the primary grafts, and to trace each invading clump of malignant cells back to the individual hyperplastic alveolus from which it has arisen. Fig. 5 shows a section through a whole graft of dorsal lobe epithelium fixed 4½ weeks after implantation. There is very little foreign body reaction, and it will be noted how clearly the foci in which malignant changes first arise may be detected. Adjacent to alveoli lined with a low columnar epithelium and distended with secretion containing epithelial debris and polymorphs are collapsed alveoli in which the epithelium has entered an exhaustion phase. The tall columnar cells, which are characteristic of collapsed alveoli, are folded and free of secretion probably owing to absorption by the host tissues.

Examination of grafts of various ages after implantation has shown conclusively that neoplasms are derived only from the epithelium which has entered the exhaustion phase of the secretory cycle. It is in these alveoli that hyperplastic epithelial changes are first seen (Fig. 8 and 9), following a phase of mitosis, abnormal cell division and pycnosis (Fig. 8). The hyperplasia is prominent in grafts four to five weeks old, giving an appearance to the epithelium which is closely similar to that seen in the human prostate during benign enlargement (Fig. 6 and 7). In no single instance in any of the numerous grafts examined has the actively secreting epithelium lining distended alveoli been the focus of neoplastic change. Presumably the non-secreting exhausted alveolar cells are more susceptible to the action of the carcinogen (Fig. 6). The hyperplastic changes in the mouse prostate are invariably accompanied by a pronounced increase in the fibromuscular stroma, and there is lymphocytic infiltration which varies considerably in different grafts of the same age (Fig. 8). In grafts five to eight weeks after implantation the alveolar epithelium contains patches which are 10–20 cells in depth (Fig. 10), with cellular proliferation predominant in the basal layers.

At this early stage it is possible to distinguish three distinct types of epithelial proliferation (A, B and C), which are illustrated respectively in Fig. 11, 12 and 13.

Type A.—Gives rise to a clump of darkly staining cells in which outlines are indistinct and there is considerable variation in nuclear size and structure (Fig. 11).

Type B.—Consists of pale staining cells undergoing squamous metaplasia (Fig. 12).

These two types of invasive new growth arise from alveolar epithelium.

Type C.—This more uncommon variety, characterized by a stratified squamous metaplasia of the epithelium *in situ*, followed by diffuse marginal growth into the stroma, clearly arises from duct epithelium (Fig. 13).

By studying the cytology and mode of growth of these early invasive cells

through later phases of development in older grafts, it was possible to predict the types of tumours which would subsequently have arisen. The tongue-like colony of early malignant cells of Type A was typical of those tumours which finally developed into secreting glandular carcinomas. In the example illustrated the proliferation arose from a single alveolus and was the only focus of malignant change in this particular graft. Later stages from other grafts showed the onset of secretory activity, and from the main focus of proliferation malignant glandular cells were seen to migrate independently into the stroma, and finally to give rise to atypical alveoli along the margins of the tumour. The newly differentiated alveoli contained irregular low columnar epithelium actively engaged in secretion. Fig. 14 shows three malignant alveoli in the peripheral area of an invading new growth of this type from an eight weeks' old graft. Several epithelial cells are in process of abnormal cell division. The lumina are filled with secretion and cell debris. Portions of this particular primary glandular carcinoma, transplanted into mice of the same age and sex as the host mouse, retained their histological characters for 16 generations of serial transplants before finally transforming into a squamous-celled tumour.

The Type B lesion arose in the various grafts examined from a single alveolus, and the example illustrated in Fig. 12 was again the only neoplastic focus within the entire graft. The proliferating cells are confined to a relatively restricted area of the alveolar wall; elsewhere the epithelium is normal in structure. The malignant cells contain small nuclei with numerous atypical mitotic figures. Apart from the different morphology and staining reactions of the proliferating cells, it will be noted that they take origin from an alveolus in which secretory activity has been inhibited. Adjacent alveoli not showing these proliferative changes are distended with secretion. In older grafts the Type B proliferation leads to the formation of pseudo-alveoli at the margins of the tumour and, although the glandular architecture of the epithelium is thus partly restored, there is no evidence of secretion. The cells acquire the characteristic intercellular fibrils of "prickle" cells, and keratinization ensues at the periphery of each alveolus. From study of many examples of this type of growth in grafts of various ages, it seems clear that the Type B proliferation leads to the formation of a squamous-cell carcinoma in which alveolar formation is preserved.

It is unnecessary to give a detailed account of the Type C proliferation (Fig. 15), except to point out that the squamous cell carcinoma derived from it grows rapidly and extensively in the grafts, and never gives rise to pseudo-alveoli. Instead the usual cell nests are formed and keratinization results in the formation of keratin pearls. It is interesting to note that the epithelium of the ductus deferens remains unaffected where it is included in the original grafted fragment, and this may be due to the thick circular muscle coat resisting the diffusion of the carcinogen (Fig. 15).

A minority of the grafts examined showed separate foci of Type A and Type C proliferation within the same graft. Fig. 16 shows an early secreting adeno-carcinoma together with a squamous-cell carcinoma arising in the wall of an adjacent duct. There is some evidence that where the two varieties of malignant change co-exist in a primary graft the subsequent tumour becomes a squamous-cell carcinoma, which rapidly infiltrates the adeno-carcinomatous areas until all trace of the glandular tumour is lost.

Influence of Bilateral Orchidectomy on Tumour Growth and Subsequent Treatment with Testosterone Propionate.

Glandular carcinoma.

An actively secreting glandular carcinoma, already in its 12th generation of serial transplants, exhibiting no evidence of squamous differentiation, was selected to determine the influence of orchidectomy on tumour growths. The castrated and the intact normal control mice all received subcutaneous transplants from

EXPLANATION OF PLATES.

FIG. 1.—Photograph of a male R III mouse after twelve months' treatment with oestrogen. Application of the sex hormone by painting the skin on the nape of the neck has induced a pronounced metaplasia of the prostatic epithelium in the dorsal and ventral lobes, causing urethral obstruction, retention of urine and bilateral hydronephrosis.

FIG. 2.—Section through the dorsal prostate of a normal Strong A mouse, showing the manner in which the glandular epithelium is thrown into folds. The secretion of the mouse prostate gland is evenly dispersed and lacks concretions. Compare with that of human gland seen in Fig. 3 × 64.

FIG. 3.—Normal human prostate gland. The secretory epithelium like that in the mouse gland is thrown into folds which project into the glandular lumina. Note alveolar concretions, and compare with that of mouse in Fig. 2. × 64.

FIG. 4.—Implant of portion of dorsal epithelium from a normal Strong A mouse without carcinogen after surviving subcutaneously in a host male mouse of same age and strain for 12 weeks. Note distension of alveoli with prostatic fluid which is far in excess of what is typical of the normal gland *in situ*, and frequently leads to cystic dilatation. Compare with Fig. 2. × 27.

FIG. 5.—Section through a whole graft of dorsal lobe epithelium impregnated with 20-methylcholanthrene fixed after 4½ weeks' subcutaneous implantation in a host mouse. There is very little foreign body reaction to the carcinogen. Note hyperplastic alveoli, adjacent to which are alveoli lined with low columnar epithelium and distended with secretion. × 20.

FIG. 6.—Hypertrophy of prostatic epithelium obtained from dorsal lobe of a Strong A mouse, impregnated with carcinogen and fixed after 4½ weeks' subcutaneous growth in a host mouse of same age, sex and strain. Also note increase in the fibro-muscular stroma. × 75.

FIG. 7.—Simple hypertrophy in a human prostate. Compare with Fig. 6. × 63.

FIG. 8.—Enlarged portion of prostatic epithelium from section illustrated in Fig. 6. As the result of carcinogenic action, several epithelial cells are seen in division, some showing abnormal mitosis and pycnosis. × 260.

FIG. 9.—Early hyperplastic changes in prostatic epithelium of a Strong A mouse, after eight weeks' subcutaneous implantation with carcinogen in a host mouse of same sex, age and strain. The glandular epithelium contains patches which are 10–20 cells in depth. × 64.

FIG. 10.—Same as Fig. 9, showing hyperplastic area of alveolar epithelium 10 to 20 cells in depth, with cellular proliferation predominant in the basal layers. × 160.

FIG. 11.—Type A lesion. Epithelial proliferation in a subcutaneous graft of dorsal prostatic epithelium impregnated with 20-methylcholanthrene fixed after eight weeks' growth in a host mouse. This early malignant tongue of cells indicated by arrows has arisen from the glandular epithelium of an adjacent alveolus, and has invaded the fibromuscular stroma of the graft. × 105.

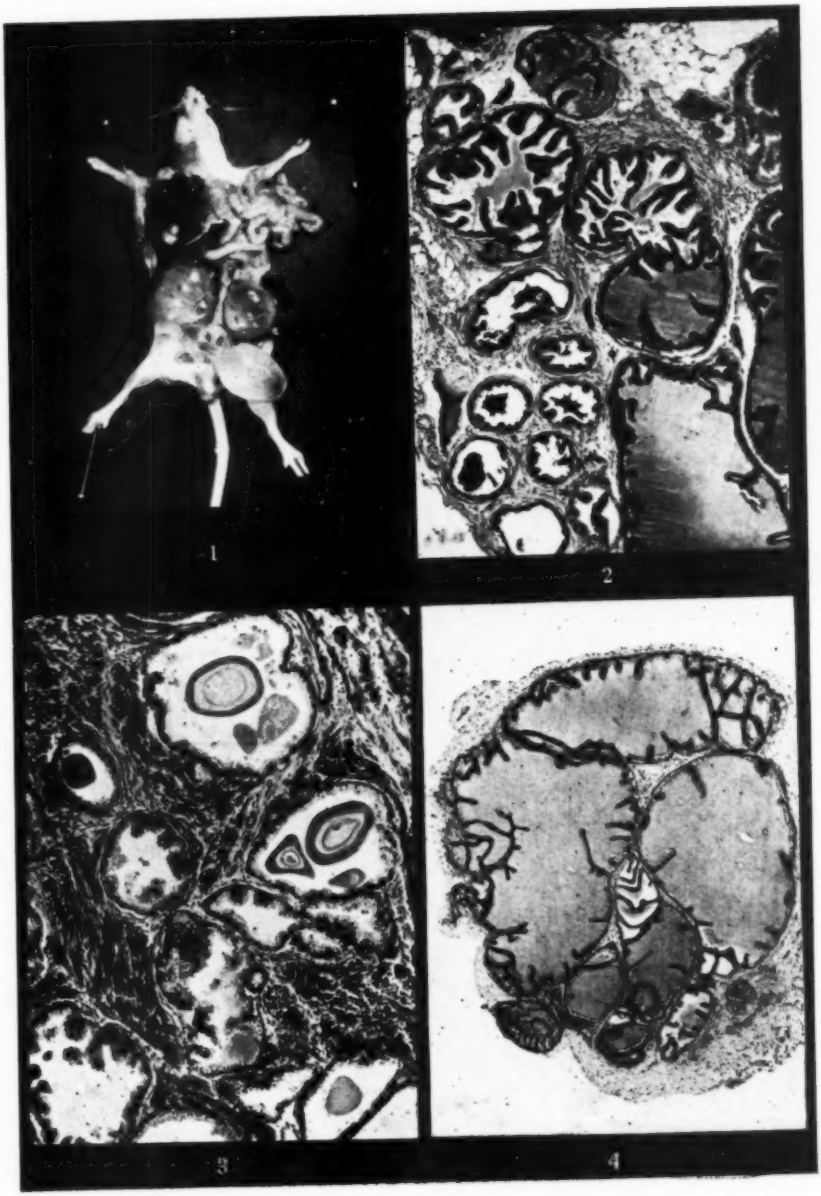
FIG. 12.—Type B lesion arose from the epithelium of a single alveolus in a subcutaneous graft impregnated with carcinogen, and fixed after 8½ weeks' growth. The proliferating cells (indicated by arrows) arose from a relatively restricted area of the alveolar wall, and from an alveolus in which secretory activity had been inhibited. × 115.

FIG. 13.—Type C lesion (illustrated by arrow) characterized by a stratified squamous metaplasia of the epithelium *in situ*, which has arisen from duct epithelium. × 53.

FIG. 14.—Three newly differentiated alveoli, consisting of irregular low columnar epithelium engaged in secretion. These malignant alveoli are in the peripheral area of an invading new growth of an eight-weeks-old graft. × 445.

FIG. 15.—Eight-weeks-old graft of dorsal lobe, showing early malignant changes in many alveoli. Note the epithelium of the ductus deferens (indicated by arrow) remains unaffected by action of the carcinogen. × 80.

FIG. 16.—7½-weeks-old graft of dorsal epithelium, with two varieties of malignant change co-existing in primary graft. One arrow indicates a squamous-cell carcinoma arising in the wall of a duct, and two arrows indicate an early glandular carcinoma. × 75.



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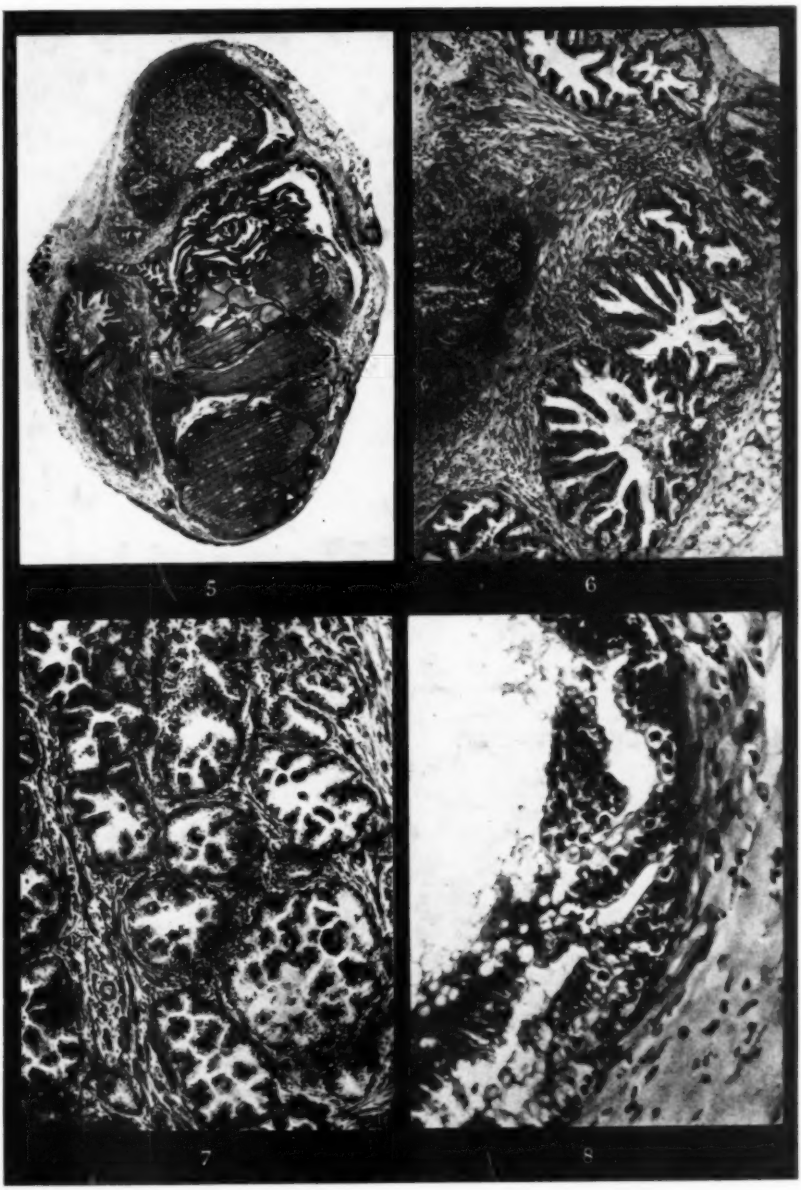

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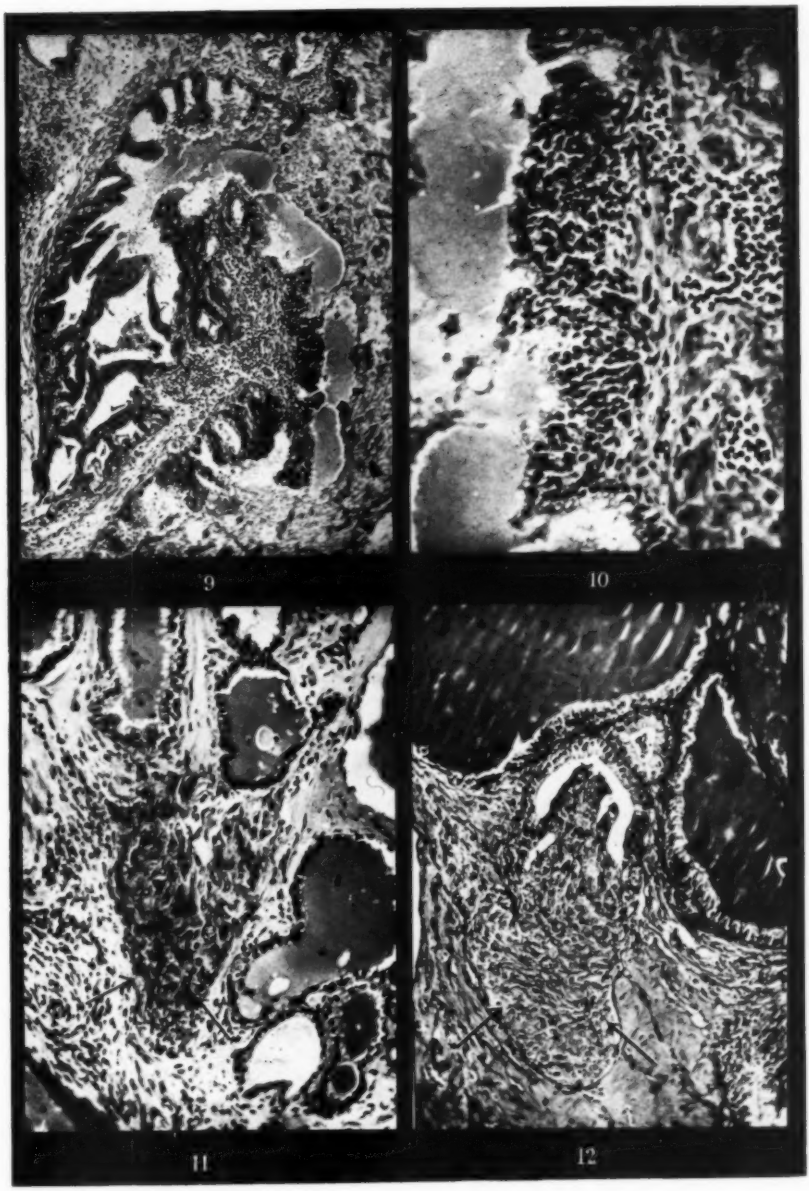

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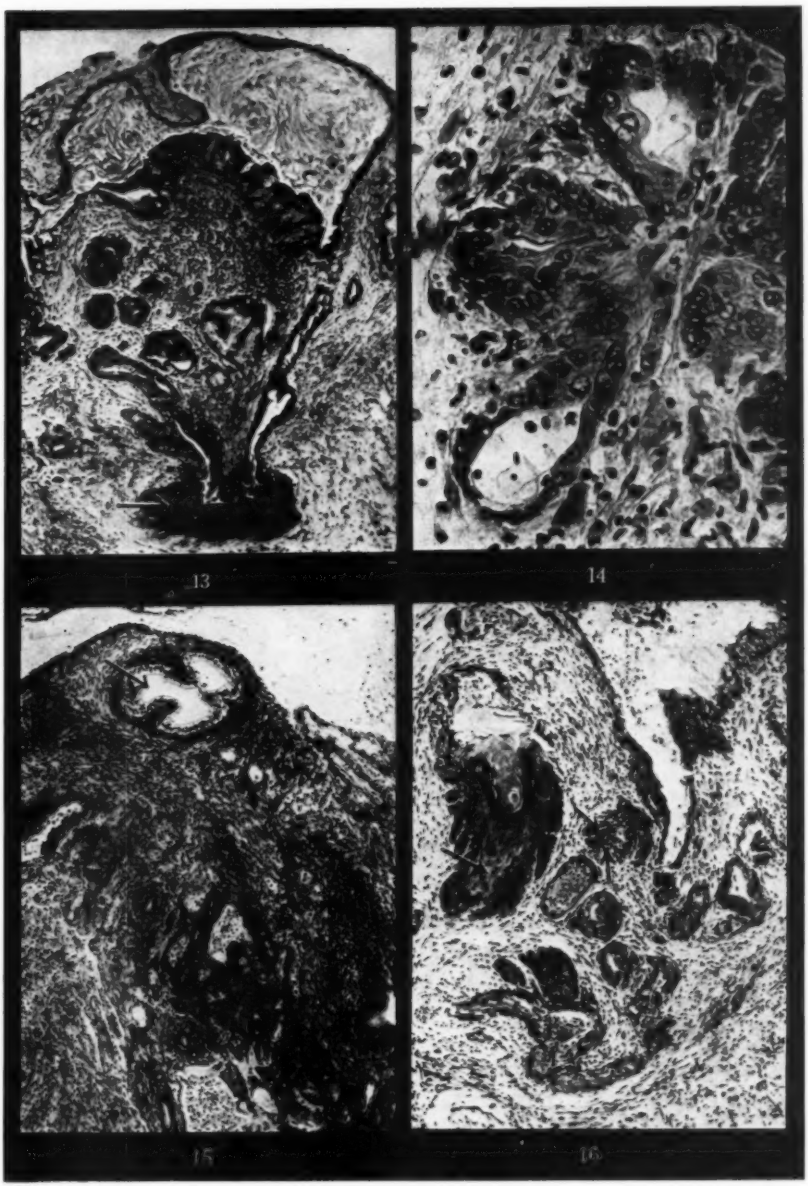


Homing.

the same tumour. All glandular tumours growing in intact normal mice showed a remarkably constant growth rate, and there was no tendency to regress throughout 14 generations of routine serial transplantation. In fact, owing to the rapid growth of this particular prostatic carcinoma, the tumour-bearing host mice either died or had to be killed during the 6th to 8th week following transplantation. Examination of the silhouette charts demonstrates the even and typical progressive growth of this tumour in the control group (Fig. 17).

All transplants grew during the first week in the castrated mice, but in the second week there was a slight inhibition of growth in comparison with those in

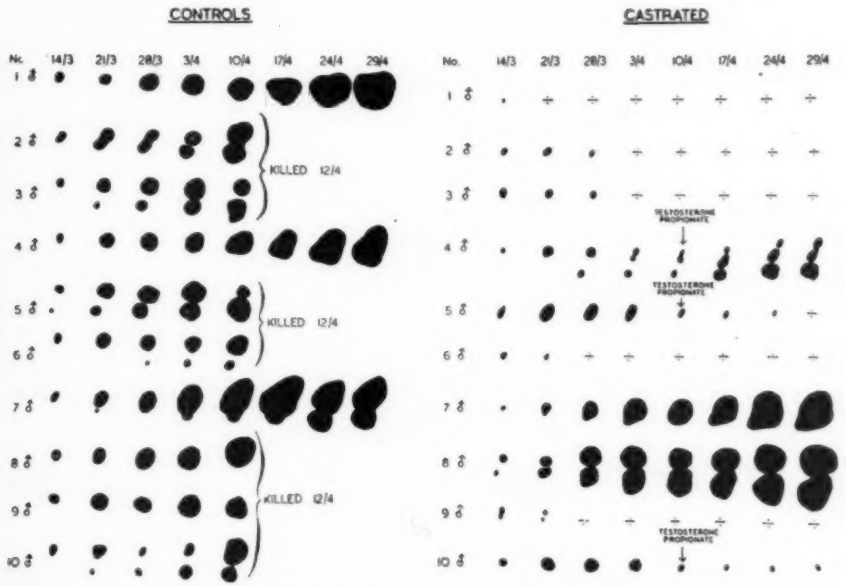


FIG. 17.—Silhouette chart illustrating the influence of bilateral orchidectomy, and of subsequent treatment with testosterone propionate, on the growth of transplantable glandular cell carcinomas of the prostate in Strong A mice. The tumours recorded here were in their 12th transplant generation.

the control group. One tumour (Fig. 17, No. 1) had completely regressed. By the end of the third week all tumours, with the exception of two which were growing at approximately the same rate as those in the controls, were rapidly regressing. On palpation the tumours undergoing regression were hard and nodular ; the two which failed to respond to orchidectomy were soft, and similar to those growing in the control mice. At the end of the 5th week five tumours had completely regressed, and three had ceased to exhibit any signs of growth (Fig. 17, No. 1, 2, 3, 6 and 9).

In order to determine the influence of the male sex hormone on the behaviour of tumour growth, the three mice still bearing regressing tumours at the end of the 5th week received every 24 hours a subcutaneous inoculation of testosterone

propionate. Two tumours failed to respond to this treatment (Fig. 17, No. 5 and 10), and the remaining tumour, after an interval of one week, began to grow

FIG. 18.—Silhouette chart showing the effects of castration on a transplantable squamous cell carcinoma of the prostate in its 12th transplant generation in Strong A mice. This tumour was originally a glandular cell carcinoma which had undergone a spontaneous metaplasia during the 10th generation of serial transplantation.

again at a rate typical of the control tumours (Fig. 17, No. 4). This particular tumour when transplanted into host mice of the same sex, age and strain was carried on in serial transplants for six generations.

The variable influence of orchidectomy on the behaviour and growth of this glandular carcinoma was further demonstrated in another series of experiments which were repeated at a later date. In this instance two groups of mice, 10 in each, likewise castrated before puberty, received transplants of the same tumour. The growth rate of three tumours in the first group of 10 mice was entirely unaffected by castration. Four tumours in the second group also grew at approximately the same rate as the transplants in the intact control mice. Of 13 tumours out of a total of 20 in the two groups which responded to orchidectomy, 4 completely regressed, and the remaining 9 tumours exhibited marked inhibition of growth. A total of 6 mice, 3 in each group, whose tumours were retarded by orchidectomy, received similar inoculations of testosterone. Four tumours responded to this treatment by showing an immediate increase in growth rate while the remainder were unaffected.

Squamous-cell carcinoma.

A glandular cell carcinoma which had undergone spontaneous metaplasia after its 10th generation of serial transplants in non-castrated normal male mice was chosen to determine the possible influence of orchidectomy on a glandular tumour which was undergoing squamous transformation. Microscopic examination of this particular tumour had shown that the metaplastic alveolar epithelium was columnar and much of it still retained secretory activity, the alveoli being distended with secretion and containing isolated epithelial cells undergoing atypical mitosis. A variable degree of keratinization associated with prickle cell transformation of the epithelium occurred in concentric nests of cells in the peripheral alveoli of the tumour.

Comparison of the growth rates of this particular tumour transplanted into intact control mice, and into castrated mice, showed that orchidectomy had very little influence on the growth of a glandular carcinoma if squamous metaplasia with keratinization had already commenced in the tumour at the beginning of the experiment (Fig. 18). The occasional slight inhibition of tumour growth in some orchidectomized mice was within the limits of possible normal variation.

The histological changes accompanying variations in growth and behaviours in castrated and non-castrated mice, together with the endocrine factors involved in this process, will be discussed in another section of this paper.

Influence of Stilboestrol Treatment on Growth.

A glandular carcinoma in its fifth generation of serial transplants and a squamous cell carcinoma in the sixth generation were selected for experiments with stilboestrol, and all tumours were transplanted into the left flanks of host mice. Pellets containing 1 mg. of diethylstilboestrol were implanted subcutaneously into the right flanks, the same dose being repeated every 14 days. Microscopical diagnosis of the tumours prior to transplantation showed that the glandular carcinoma was a fairly well differentiated and actively secreting tumour. The second type of tumour, since its induction as a primary new growth, had maintained its structure as a squamous cell carcinoma during serial transplantation and was not originally a glandular tumour which had undergone squamous metaplasia. From its first appearance this tumour exhibited prickle cells, keratinization and a deficiency of stroma.

Glandular cell carcinoma.

The influence of stilboestrol on this particular tumour was determined for a period of six weeks on a total of 50 mice. There were five separate experiments, each comprising 10 treated mice, with equal numbers of untreated controls. Fig. 19 shows the effects of the oestrogen on tumour growth in a batch of 10

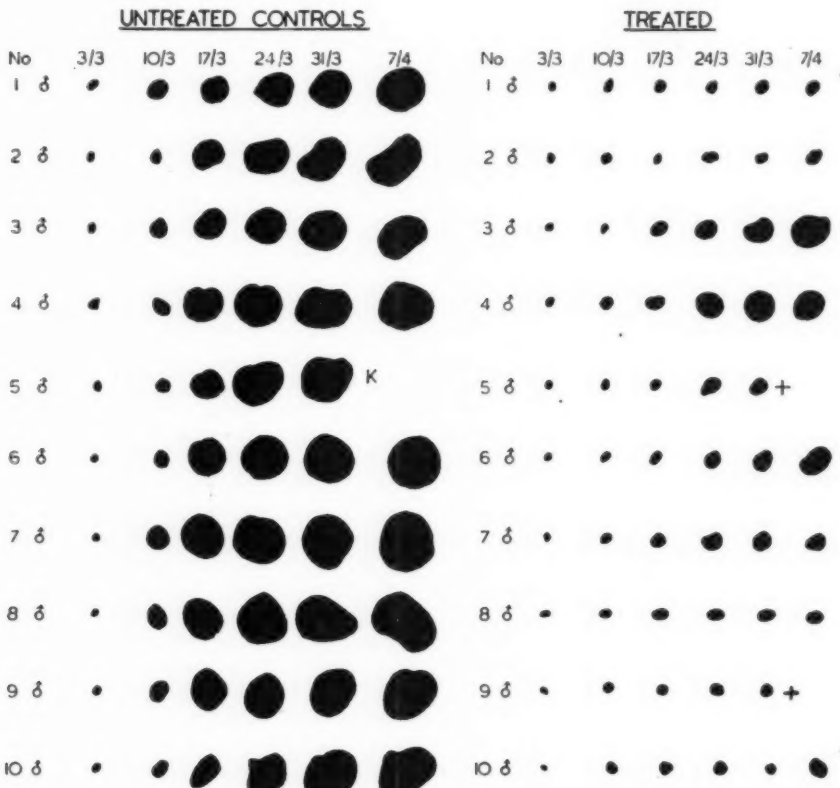


FIG. 19.—Silhouette chart illustrating the influence of stilboestrol treatment on a glandular carcinoma of the prostate in its 6th generation of serial transplantation. K. = killed. + = died.

mice, and is representative of the general effect obtained throughout. Of 50 treated mice 38 showed a slight arrest of tumour growth, 15 displayed a more pronounced retardation, whilst the remaining tumours still continued to grow at approximately the same rate as in the controls. In no single instance did any of the treated tumours undergo a complete regression during the six weeks' period of treatment. Although the first dosage of stilboestrol did not appear to impair the health of the mice, at the end of three weeks' treatment the average loss of

weight was approximately 0.25 g. Thus, as will be clear from Fig. 19, although stilboestrol had in most cases retarded the growth rate of the tumours and there was a fall in body weight; no single tumour showed a complete regression similar to that obtained in some instances when the host mice had been castrated previous to transplantation. The continuation of stilboestrol administration led to further loss in body weight; some mice died and the rest became too unhealthy

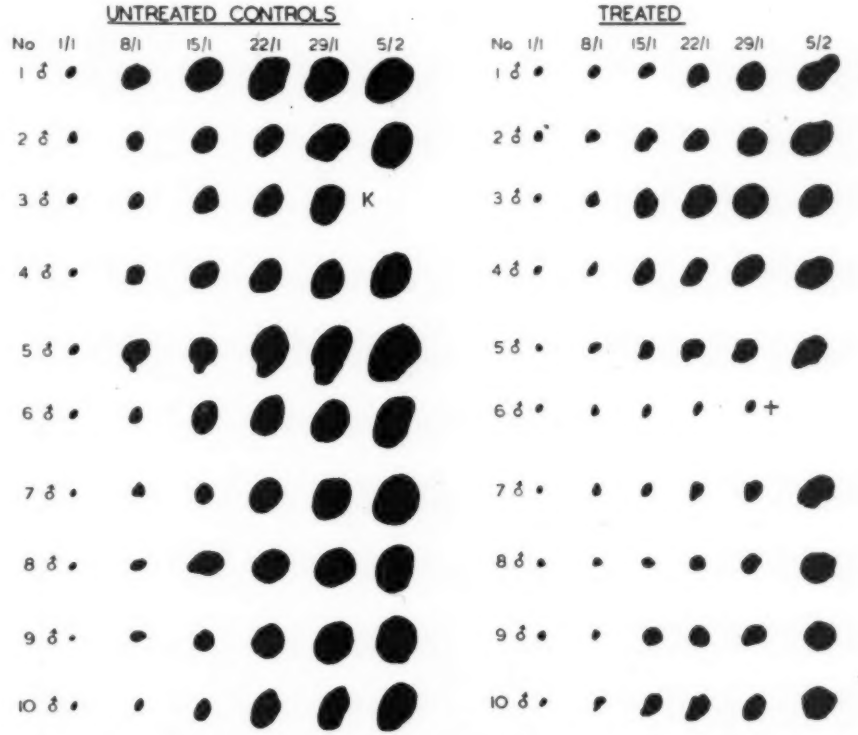


FIG. 20.—Silhouette chart. A squamous cell carcinoma of the prostate in the 7th transplant generation, showing the effects of stilboestrol treatment. K. = killed. + = died.

for reliable estimation of the responses of the tumours. On palpation these were found to be hard and nodular, as compared with the tumours in untreated mice, which were soft and spongy.

Squamous cell carcinoma.

Unlike the glandular cell carcinoma none of the squamous tumours showed any pronounced sensitivity to stilboestrol treatment (Fig. 20). The same numbers of mice were used of similar age and treated with stilboestrol for the same duration. There was a delay, in comparison with the response of the glandular tumours, of

three weeks before any effect of the oestrogen could be detected. Then approximately three out of ten in each of the five groups of mice showed a slight arrest of tumour growth. As these squamous tumours are normally hard and firm, no apparent change could be detected by palpation after four weeks' administration of oestrogen. By the sixth week the loss of body weight amounted to 1 g., and some mice had already died during the previous week.

Histological Changes Induced by Castration and by Stilboestrol Treatment.

Castration.

Glandular carcinomas.—The tumours examined in greater detail have been selected according to their response to castration alone, or to castration followed by administration of testosterone propionate. Cytological examination was made eight weeks after transplantation.

Tumours exhibiting no inhibition of growth following castration, e.g. No. 7 in Fig. 17, were harder and more nodular on palpation than those growing in control intact mice. Secretory activity as judged from sections had been suppressed and squamous metaplasia, of variable intensity in different areas of any tumour, was always found. The prostatic alveoli still retained their characteristic form and, although secretion appeared to be inhibited, many lumina contained prostatic coagulum and cell debris. Keratinization was restricted to cells at the periphery of the alveoli (Fig. 21). In Tumour 8, Fig. 17, there was no keratinization, but many epithelial cells possessed intercellular fibrils.

Tumours showing considerable regression of growth in response to castration underwent more pronounced cytological changes. Thus in the extreme case of Tumour 5, Fig. 17, which had completely regressed and had shown no response to testosterone, islands of degenerating epithelium lying in a stroma which was largely replaced by keratin formed the characteristic histological picture. Epithelial degeneration was almost complete (see Fig. 22), and the interior of the

EXPANATION OF PLATES.

FIG. 21.—Tumour 7 in Fig. 17, showing suppression of secretory activity, squamous metaplasia and keratinization of cells at periphery of alveoli in a glandular carcinoma following transplantation into a male mouse castrated before puberty. Although this tumour exhibited no inhibition of growth, it underwent pronounced histological changes during transplantation. × 390.

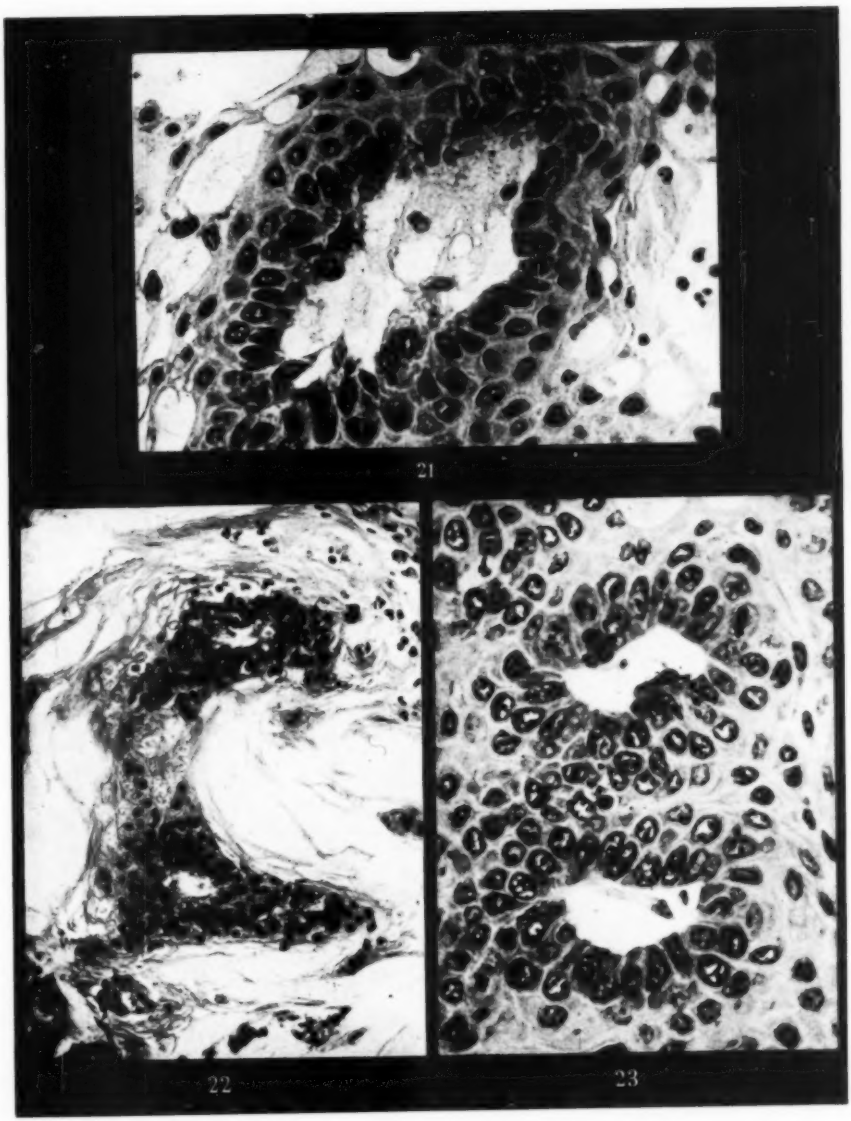
FIG. 22.—Tumour 5 in Fig. 17, illustrating a tumour which had completely regressed following transplantation into a castrated mouse, and failed to resume growth following treatment with testosterone propionate. Epithelial degeneration is complete, and interior of tumour consists largely of keratin. × 390.

FIG. 23.—Tumour 4, Fig. 17. The alveolar character of the glandular carcinoma persists in this tumour, which has undergone squamous metaplasia after transplantation into an orchidectomized mouse. This tumour regressed in response to castration, and resumed growth following treatment of host mouse with testosterone proprionate. × 390.

FIG. 24.—Alveolus of a glandular carcinoma eight days after treatment of host mouse with stilboestrol. There is a complete inhibition of secretory activity of glandular epithelium, and a slight shrinkage of nuclei. × 450.

FIG. 25.—Alveolus of a glandular tumour 3½ weeks after commencement of treatment of host mouse with stilboestrol. Note the vacuolation (indicated by arrows) of secretory epithelium, which is largely confined to the basal region of epithelial cells. Several nuclei have undergone chromatolysis. There is also a complete inhibition of secretory activity. × 450.

FIG. 26.—Alveolus of a tumour which responded to treatment five weeks following administration of stilboestrol. There is a marked reduction in height of epithelium, and most nuclei are pycnotic. The vacuolation has resulted in rupture of apical cell membrane, with expulsion of cell contents into the lumina of the alveoli. × 340.



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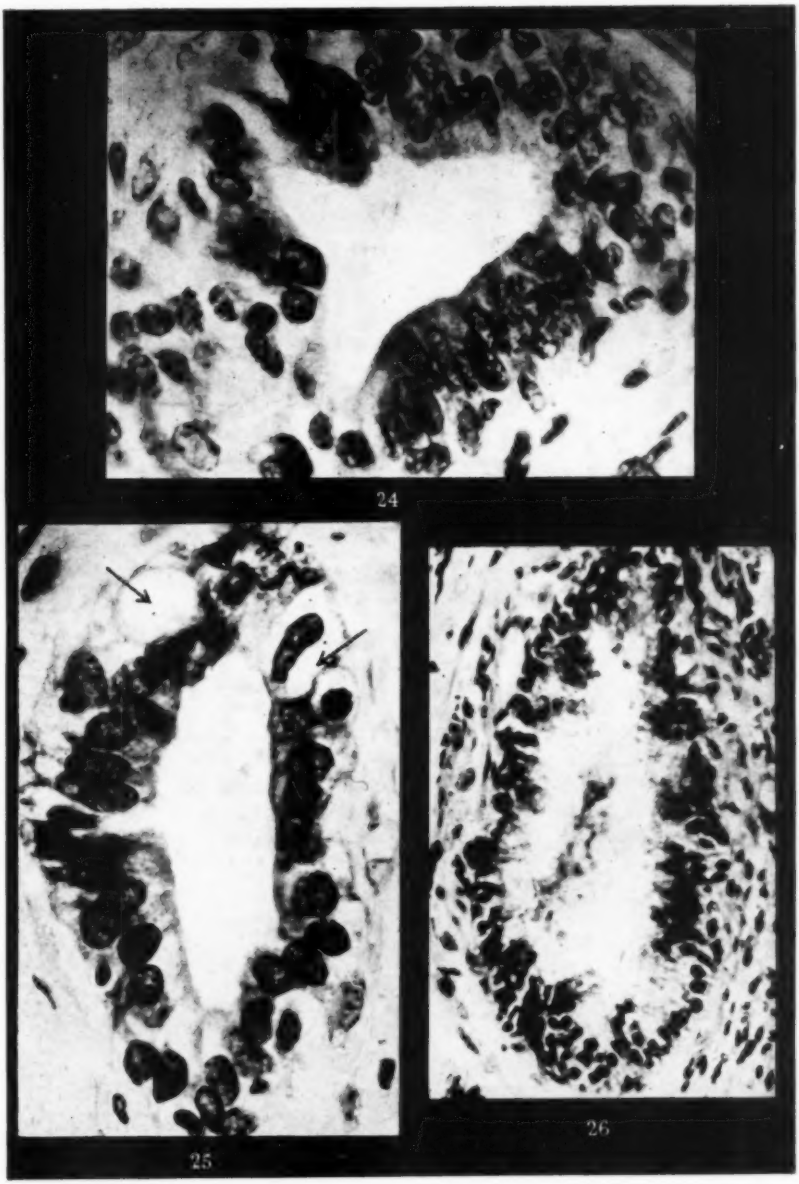

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tumour consisted largely of keratin. There was, of course, no mitotic activity and in fact the cells were shrunken to scanty fringes of cytoplasm containing nuclei in advanced stages of chromatolysis. Portions of this tumour failed to grow when transplanted into normal host mice.

Tumours inhibited by castration but subsequently treated with testosterone, after which growth was renewed, were examined at the end of the third week of hormone administration, e.g. Tumour 4, Fig. 17. A similar squamous metaplasia with inhibition of secretion was found together with considerable mitotic activity (Fig. 23). The alveolar character of the growth had persisted, but the epithelial cells were shorter than in the original untreated tumour. These results were confirmed in a second series of experiments involving the same number of castrated mice using the same dosages of testosterone. There was abundant mitotic activity in these tumours responding to testosterone, and these grew successfully for six generations when transplanted into normal male mice. The squamous character of the tumour was retained with a uniform growth rate until the conclusion of the experiment. The tumours which failed to respond to testosterone had undergone extreme keratinization, and the epithelial islands consisted as before of degenerating cells (Fig. 22).

Squamous cell carcinomas.

Tumours which from the time of their induction had been diagnosed as squamous cell carcinomas were similarly transplanted into mice castrated before puberty. Examination of Fig. 18 shows that castration had no appreciable influence on squamous tumours of this type. There were no specific cytological changes except in those tumours (No. 2, 5, 9 and 10, Fig. 18) in which a slight retardation of growth coincided with patches of keratinization leading to the formation of keratin pearls.

Stilboestrol treatment.

Glandular carcinoma.—Unfortunately stilboestrol has a toxic effect on this special strain of mice, and tumour regression was in nearly every case accompanied by loss in weight of each tumour-bearing mouse (Fig. 19). A dosage of 1 mg. did not appear to impair the health of the mice until the end of the second week's treatment, after which three deaths occurred. The average loss of weight per mouse at the end of two weeks' treatment was approximately 2·5 g.

Complete inhibition of secretory activity of the prostatic epithelium occurred eight days after treatment, and following this the oestrogen appeared to induce two distinct kinds of histological change in the alveolar epithelium of this particular prostatic carcinoma: (*a*) a squamous metaplasia similar to that which has been described by several authors (Lacassagne, 1933; Burrows and Kennaway, 1934; Horning, 1947) for the prostate glands of normal intact rodents, or (*b*) a progressive vacuolation of the glandular cells, finally leading to disintegration of the alveoli. This latter type of response to treatment, though more uncommon in the rodent, is similar to that reported in man following stilboestrol treatment for prostatic carcinoma (Schenken, Burns and Kahle, 1942), and will be described in detail.

The cytological changes after varying periods of treatment all follow a similar pattern, although the latent period before onset varies slightly in individual mice. In the majority of mice, after three to four weeks' treatment, a vacuolation

occurs in the basal cytoplasm of the glandular cells, with or without stratification of the epithelium. This process is more pronounced in those tumours which show a more marked inhibition of growth. Tumours found to be retarded in growth during the fifth week of treatment were characterized by a decrease in the size of the majority of the alveoli, a reduction in the height of the epithelium, a complete disappearance of cell-division, and an increase in the fibro-muscular stroma. The nuclei had become smaller, and many were hyperchromatic and later pycnotic. The most conspicuous cytoplasmic change seen in Bouin-fixed material was the vacuolation restricted at first to the basal region of the cells (Figs. 24 and 25). In many alveoli these vacuoles varied considerably in size, and often displaced the pycnotic nuclei towards the apical end of the distorted cells. Tumour material fixed in osmium tetroxide five weeks after oestrogen treatment showed the vacuoles to contain lipid droplets. The alveoli lined by stratified epithelium of varying thickness in different tumours did not possess any appreciable epithelial vacuolation.

In most regressing tumours intensified vacuolation of the glandular cells finally resulted in the rupture of the apical cell membrane, with expulsion of the cell contents into the lumina of the alveoli (Fig. 26). The alveoli diminished in size when this rupture occurred, whereas adjacent alveoli with intact epithelial cells appeared to be so slightly affected by the oestrogen that little more than a reduction in the height of the cells could be observed. Tumours after five to seven weeks' treatment grew erratically when first transplanted into normal mice during the first three generations of serial transplants. In subsequent generations these tumours invariably assumed a constant and progressive rate of growth, as though they had overcome what was only a temporary inhibition in response to stilboestrol.

DISCUSSION.

The technique adopted in these experiments of inducing tumours of the prostate gland in mice lends itself admirably to the study of the early histogenesis of malignant formation, partly because the sites where neoplastic changes first occur are uncomplicated by foreign body reaction or necrosis within the graft. It was possible in grafts impregnated with carcinogens to follow in four to six weeks after implantation hyperplastic changes in the glandular epithelium, in which three distinct types of early malignancy could be distinguished. The types of tumours which would have developed subsequently could be predicted, even though cellular proliferations had occurred in many instances from only a single alveolus. A detailed study of the hyperplastic changes in numerous grafts has shown conclusively that in no single instance has an actively secreting epithelium been the focus of malignant change. The non-secreting exhausted alveolar cells appear to be more susceptible to the action of the carcinogen than those at the height of secretory activity. This observation supports Haddow's (1947) contention that chemical carcinogens act more readily on cells following depression of their functional activity.

It is difficult to interpret the reasons why previous attempts (Dunning, Curtis and Segaloff, 1946; Horning and Dmochowski, 1947) to obtain glandular carcinomas by injecting the prostate glands of living rodents *in situ* with carcinogens have only succeeded in inducing squamous or sarcomatous growths. Obviously there are several factors involved, and one of them might be that it is

necessary for the carcinogen to gain close contact with the prostatic epithelium without at the same time damaging it. The technique used in the present experiments is possibly superior to that involving injection of carcinogens, dissolved in such solvents as lard, merely because it entails less damage and concentrates the carcinogen at the proper site. Since the present technique also involves homologous grafting, however, the reaction between host and graft tissues may play some part in the successful induction of a tumour. In mice the survival of a homologous graft seems to depend on the use of a closely inbred strain. Stock mice of indeterminate ancestry will not often tolerate grafts for periods sufficient for the establishment of tumours. Recent experiments have indicated furthermore that the age of the mice providing the graft tissue, and the age of the host mice into which the grafts are implanted, have an important bearing on the problem. Thus the survival rate of grafts of lung or prostatic epithelium without carcinogen was greater if host mice under eight weeks old were used, and was considerably reduced if the mice were over eight months old. This was invariably the case if the mice were of mixed or pure line origin, but only 12 per cent of the grafts in young mixed stock mice survived as against 85 per cent in the young pure line mice. In older mixed stock mice of 8 months to one year no graft survived after three months' implantation, whilst in inbred mice of a similar age 25 per cent of grafts survived after the same period. Combining the carcinogen 20-methylcholanthrene with grafts of lung or prostatic epithelium in 25 eight-weeks-old mice of mixed stock gave only one tumour, a spindle-celled sarcoma, twelve weeks after grafting; the remaining 24 host mice failed to produce tumours, and at autopsy the grafts were found not to have become vascularized. Price (1941), using rats of different ages from an albino strain which was not strictly pure line, found that ventral lobe prostatic tissue grafted subcutaneously in the abdominal wall behaved in much the same way, prostatic tissues from old rats being very limited in their capacity to survive as homologous grafts. Many apparently viable grafts from old rats were found to be degenerate on histological examination, but when regrafted into younger hosts the implants underwent a rapid recovery and the prostatic epithelium even regained its secretory activity. One of the most interesting features in successful grafting of prostatic epithelium is its ability to continue secreting while growing under the influence of foreign host reactions and changed vascularity. Obviously androgenic stimulation, as Price (1941) maintains, must be important in this phenomenon.

The dependence of prostatic epithelium upon androgen secretion has long been realized and the experimental evidence need not be referred to here, except to mention that in recent years the elegant technique of transplantation of fragments of prostate into the anterior chamber of the eye has provided additional confirmation. Moore, Melchionna, Tolins and Rosenblum (1937), Moore, Rosenblum, Tolins and Melchionna (1937), and Moore and Smith (1937), grafted rabbit prostatic epithelium in this way and photographed the implants daily. They were able to record fluctuations in size of the grafts which were diminished or abolished by castration, and considerably increased by administration of testosterone propionate. Heckel and Kretschmer (1935) made similar observations of the responses of prostatic grafts to anterior pituitary extracts. Androgens from sources other than the testis can also be shown to influence the behaviour of the prostate. Thus grafts of ovary transplanted into the ears of castrated

mice or rats will maintain the size and normal histological appearances of the prostate and seminal vesicles. Hill and Strong (1938) attribute the androgenic effect of the ovary to the lowered temperature of the ear, and Deanesly (1938) has correlated this activity with extensive luteinization of the theca interna within the ovarian grafts.

Another potential source of androgens to be considered in relation to prostatic grafts and tumours is, of course, the adrenal cortex. The rare adrenal insufficiency in young children associated with adrenal hypertrophy leads to enlargement of the prostate and differentiation of its glandular tissues to a degree comparable with the adult gland (Dijkhuizen and Behr, 1939–40). Prostatic enlargement also occurs in adults with adrenal cortical tumours. Miller (1947) has found a relative increase in dehydroisoandrosterone, and a decrease in androsterone in the urine of patients with benign hypertrophy of the prostate. According to Callow and Callow (1940) the source of dehydroisoandrosterone is probably in the adrenal cortex. It remains in the urine after castration, but disappears in cases where there is destruction of the adrenal cortex.

While it might be assumed that grafts of prostate or transplantable prostatic tumours would have poor chances of survival in female hosts, under certain conditions they do in fact survive, and this must be attributed to the influence of androgens produced either in the ovary or in the adrenal cortex. Price (1941) grafted normal prostate into female host rats, and found that in some cases the grafts were similar in histological structure to the prostates of castrate males, whereas other grafts flourished and underwent secretory activity. This result she maintained could be due to the development of androgenic foci in the ovaries of some, but not all, host animals. The grafts failed to differentiate in spayed rats, whereas they survived in a functional condition for prolonged periods if the hosts were over 80 days old. Either virgin females or rats which had littered frequently would sustain functional grafts, provided they were not younger than this apparently critical age.

The opinion is often expressed that the prostate gland in rodents is not strictly homologous with the prostate in the human, and thus there can be little justification for comparing growth behaviour and endocrine responses in experimentally induced prostatic tumours or hyperplasias with the spontaneously occurring conditions in the human gland. Greenstein (1947) maintains for instance, that because certain rodent prostate tumours grow when transplanted into female mice, whereas human tumours are so sensitive to inhibition by oestrogens, the two types of neoplasm are not fundamentally the same. Apart from the single case reported by Deming, Jenkins and Van Wagenen (1935) of a spontaneous nodular hyperplasia in the suburethral tissue of an albino rat, there is no record of spontaneous prostatic hyperplasia or cancer in rodents. If it can be shown, however, that the prostate tumours induced by methylcholanthrene behave in essentially the same way to hormone administration or deprivation, the differences between rodent and human neoplasms will be less significant.

The glandular carcinomas described in these experiments, when transplanted into mice castrated before puberty, show complete inhibition of secretion, later followed by squamous differentiation. The tumours which regress quickly are more squamous in character, and subsequently atrophy by a rapid process of keratinization. Administration of androgens will cause some of these inhibited tumours in castrated hosts to resume growth if the squamous change and kera-

tinization are not too advanced. When, however, a tumour arises as a squamous-cell carcinoma in the initial graft containing the carcinogen, subsequent transplantation into castrated hosts is not followed by any appreciable inhibition. Some glandular celled carcinomas are thus dependent for their sustained growth on an adequate androgen level ; the squamous growths would appear to flourish without testicular androgens and, if they are at all androgen-dependent, it must be concluded that adrenal cortical androgens are in these cases sufficient. According to Huggins and Hodges (1941) human prostatic cancer falls into two groups, androgen dependent and androgen independent. It is possible that squamous-celled carcinomas in mice belong to the second category, although such tumours rarely occur in man (Willis, 1948).

A small percentage of glandular mouse tumours failed to regress when transplanted into castrated hosts ; they even grew at approximately the same rate as those transplanted into uncastrated mice. Howard (1937, 1938) has demonstrated that removal of the gonads in rats, provided these are sufficiently young, increases the capacity of the adrenals to secrete androgens. As all the mice in the present experiments were castrated before puberty, and an interval of several months elapsed before they received tumour transplants, it seems probable that androgenic foci had developed in the adrenals of those castrated mice in which the tumours showed no inhibition of growth. Unfortunately biochemical assay of androgens secreted from non-testicular sources cannot be undertaken in the mouse to confirm or refute these conjectures.

On the clinical side the serum acid phosphatase and urinary 17-ketosteroid levels can be used respectively as indicators of the activity of malignant prostate cells and of androgen secretion. Thus Huggins, Stevens and Hodges (1941) studied cases in which a recurrence of prostatic cancer had followed an initial improvement in the condition obtained by bilateral orchidectomy. There was a rapid reduction in the serum acid phosphatase, and a similar marked fall in the urinary 17-ketosteroids after orchidectomy. A sudden rise in output of these substances, greatly accelerated by administration of androgens, coincided with renewed growth of the temporarily inhibited tumours. The development of androgenic foci in the adrenals was regarded by Huggins and Scott (1945) as responsible for the recurrence of the disease. Recently, following failure in four selected cases to control growths by orchidectomy, these workers tried bilateral adrenalectomy and, although three of the patients died within a comparatively short time after operation, the fourth survived for 116 days. The 17-ketosteroids fell in every case to almost zero, but only slight changes were produced in the acid phosphatases. Cox (1947) tried unilateral adrenalectomy on three patients with prostatic cancer, all of whom had temporarily responded to treatment with oestrogens. There was an immediate drop in the 17-ketosteroid level and a " dramatic if only temporary improvement " in two of these cases.

The majority of the glandular tumours transplanted into normal male mice showed a varying retardation of growth after oestrogen treatment, but in no single instance did any undergo complete regression, as was the case following orchidectomy. Most mice, and according to Ludford and Dmochowski (1947), especially the strain used in these experiments, are particularly sensitive to stilboestrol. Using 2 mg. doses in the form of pellets prepared for subcutaneous insertion, these authors investigated the effects of stilboestrol on ten different transplantable tumours in four inbred strains of mice. They concluded that

inhibition of tumour growth with such relatively high doses was the result of non-specific toxic action. It was also found that the amount of stilboestrol tolerated depended upon the genetic constitution of the mice. Although in the present experiments the dose of stilboestrol was approximately half of that used by Ludford and Dmochowski (1947), the average loss of weight of the tumour-bearing mice was approximately 1½ to 2 g. during the total period of treatment. Histological examination of regressing glandular tumours after stilboestrol therapy suggested that diminution in size of the tumours was primarily due to inhibition of secretion, later followed by degeneration and collapse of alveoli. Schenken, Burns and Kahle (1942) and Fergusson (1946) have examined serial biopsies of human prostatic carcinoma under oestrogen treatment. The former authors described in detail such cytological changes as vacuolation of the alveolar epithelium, disintegration of the cell membranes, sometimes accompanied by stratification of the epithelium, and an increase in the stroma. These changes are very similar to those which occur in the glandular mouse tumours during oestrogen treatment. The increase in stroma of both human and mouse tumours recalls the experiments of Price (1941) in which a similar change occurred in grafts of normal rat prostate from the ventral lobe when implanted subcutaneously into female host rats. The squamous metaplasia, which some of the glandular mouse tumours undergo in response to oestrogen administration, is rarely found in the human gland after similar treatment. Recently, however, Inglis (1948) described a pronounced squamous differentiation in a man treated with stilboestrol for prostatic cancer.

It is tempting to regard the atrophy of prostatic tumours in the mouse as due to failure of optimum androgen secretion in the castrated hosts, or to a similar drop in gonadotrophin production when intact tumour-bearing mice are treated with oestrogens. Those tumours which fail to regress are possibly receiving sufficient androgenic stimulation from the adrenal cortex. Inhibition of secretion and degeneration of the epithelium in these tumours are so similar to the changes induced in the normal prostate following castration or oestrogen administration that any more complicated mechanism operating in the case of the tumour-bearing mice seems unnecessary. The squamous metaplasia, which in some cases is present when a tumour is first induced, does not invariably occur in all tumours even after repeated transplantation, and remains a puzzling feature of the problem. Dean, Woodward and Twomby (1947), however, have criticized the theory of Huggins and Hodges (1941) that castration and stilboestrol both exert their effects on human prostatic cancer by inhibiting androgen output, since they claim that castration raises the 17-ketosteroid excretion, whereas stilboestrol depresses it. Preliminary experiments on the effects of castration in dogs by Amoroso, Gough and Horning (1949) have shown an initial fall, not a rise in 17-ketosteroids. Since investigation of the hormone output in mice is impracticable it will obviously be necessary to collect data from a more suitable animal like the dog, in which it is hoped that a transplantable glandular tumour may be obtained.

SUMMARY.

A technique for the induction of prostatic glandular carcinomas in mice arising from subcutaneous grafts of prostatic epithelium impregnated with 20-methylcholanthrene is described, and the factors upon which homologous

grafts are dependent for their successful survival and growth in host animals are discussed.

Because this method of tumour induction permits the study of the early histogenesis of malignant formation, as no residue or necrosis within the grafts conceals the actual areas where neoplastic changes first arise, it has been possible in grafts, impregnated with the carcinogen, as soon as four to eight weeks following implantation, to distinguish between three distinct types of early malignant lesions.

Detailed study of the hyperplastic changes in prostatic alveoli in numerous grafts showed that in no single instance has the actively secreting epithelium been the focus of malignant change. It therefore appears that the non-secreting exhausted alveolar cells in the prostatic gland are more susceptible to the action of the carcinogen than those at the height of secretory activity. This supports the contention that chemical carcinogens act more readily on cells following depression of cellular activity.

The influence of sex hormones and orchidectomy was determined on the behaviour and growth of glandular and squamous-cell carcinomas of the prostate. A greater number of glandular carcinomas regress when transplanted into male mice castrated before puberty, and a small percentage of these tumours resume growth when treated with testosterone propionate. Tumours which at the time of their induction were diagnosed as squamous growths, or glandular carcinomas which had undergone spontaneous squamous differentiation during serial transplantation, exhibited no appreciable response to these forms of therapy. These results indicate that only glandular and not squamous carcinomas are dependent for their sustained growth on an adequate androgen level.

Glandular carcinomas when treated with diethylstilboestrol respond more readily than squamous tumours. The cytological changes induced in prostatic tumours during treatment by orchidectomy and oestrogen administration have been described, and have given additional information on the factors responsible for tumour regression.

Particular attention has been paid to those glandular tumours which failed to respond to either orchidectomy or hormonal theraphy, and the possible endocrine factors inducing resistance to treatment are discussed in the light of recent experimental and clinical evidence.

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MAMMARY TUMOURS IN HYBRID MICE: THE PRESENCE AND TRANSMISSION OF THE MAMMARY TUMOUR AGENT.

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A PREVIOUS paper (Foulds, 1947) recorded the origin in hybrid mice of spontaneous mammary tumours, many of which were transplantable in female or oestrogenized male hosts but not in normal males. The present paper is concerned primarily with evidence for the presence and transmission of the mammary tumour agent. Two succeeding papers (Foulds, 1949*a*, *b*) deal with the responsiveness of transplanted and of spontaneous mammary tumours to hormones.

The hybrid mice which provided the hormone-responsive transplantable

tumours were obtained by mating females of the low mammary tumour strain C57 black with males of the high mammary tumour strain R3 and were described briefly as CR hybrids. To conform with the more usual practice of using "B" as the abbreviation for C57 black and to avoid confusion with the "C" strain used by American workers, the designation BR F_1 is now applied to the (C57 black ♀ × R3 ♂) F_1 hybrids and RB F_1 to the reciprocal hybrids (R3 ♀ × C57 black ♂) F_1. Thus BR and RB correspond with CR and RC respectively in the earlier paper.

The present investigations provide evidence for the presence of tumour agent in BR F_1 hybrids, although these mice were nursed by their C57 black mothers presumed deficient in tumour agent. They show that spontaneous mammary tumours developed abundantly in the progeny of two tumour-bearing BR F_1 females, and that extracts of a transplanted BR F_1 tumour induced the growth of mammary tumours in other hybrids deficient in the tumour agent. Further observations on BR F_1 hybrids are recorded, and a high incidence of tumours in the reciprocal RB F_1 hybrids is described. Finally it is shown that the incidence of tumours in BR F_1 hybrids is greatly reduced or delayed when the male parent is derived from a line of R3 mice freed from the tumour agent.

Mammary tumours in BR F_1 mice.

Amongst the survivors of the BR F_1 colony described in the earlier report (Foulds, 1947), three breeding females and two virgins subsequently developed tumours. Two breeding females had typical multiple tumours appearing at ages of 64 and 66 weeks respectively. The remaining breeding female and the two virgins had tumours of atypical structure; the tumours in the breeding female and that in one of the virgins developed at or near the point of needle entry for an intraperitoneal injection of an oily solution of a carcinogen and, therefore, were not certainly spontaneous growths.

The BR F_1 hybrids were not bred primarily for the determination of mammary tumour incidence, and the records are unsatisfactory for that purpose. Some mice received intraperitoneal injections of carcinogens whose contribution to the development of mammary tumours was difficult to assess; others were used for transplantation experiments. The mice which survived to tumour age, though not themselves used for experiment, were not certainly representative of the whole colony and, owing to the concentration of tumours in families, the inclusion or omission of a single litter could greatly alter the tumour incidence calculated on the small numbers available. Consequently only a tentative estimate of the mammary tumour incidence is practicable. Of the original colony of 71 breeding females, 11 (15 per cent) developed typical mammary tumours at an average age of 40 weeks (range 26 to 66 weeks); 7 of the mice were "normal," 3 had received intraperitoneal injections of a carcinogen and 1 a tumour implant. Three out of 22 virgins developed tumours. A typical tumour was found at the age of 55 weeks near the site of injection of a carcinogen, 1 atypical tumour at the age of 81 weeks also near the site of injection of a carcinogen, and another atypical tumour at the age of 110 weeks in a "normal" virgin.

The breeding of BR F_1 hybrids was continued, from other parents, mainly to provide hosts for transplantation experiments. Only a minority of mice

survived to tumour age and no estimate of the mammary tumour incidence is possible. Typical mammary tumours developed in 12 breeding females at an average age of 36 weeks (range 26–64 weeks). Seven of the tumour-bearing mice were from three litters containing respectively 3, 2 and 2 tumour-bearing sisters. It was evident that the occurrence of mammary tumours in the earlier series was not fortuitous.

It was concluded that a low or moderate incidence of mammary tumours in BR F_1 hybrids was accountable to a high incidence of mammary tumours in a minority of families, the majority of families being tumour-free. The breeding experiments described in the next section were designed to test the hypothesis that the BR F_1 hybrids were heterogeneous in their capacity for mammary tumour production.

Mammary tumours in the progeny of BR F_1 mice.

Progeny were obtained from 2 tumour-bearing BR F_1 females, BR4 and BR6 (bearers of the tumours CR4 and CR6 recorded in the earlier paper (Foulds,

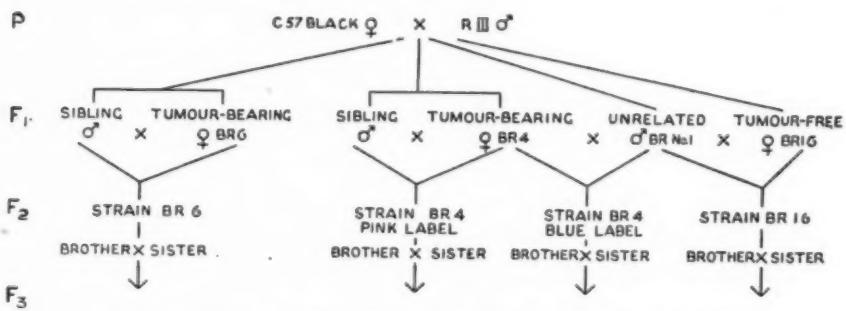

FIG. 1.—Scheme of breeding from tumour-bearing and tumour-free hybrids.

1947)), and from 1 female, BR16, which had a lung tumour but no discernible mammary tumour when killed at the age of 130 weeks.

Mouse BR6 was mated with a sibling male to provide the F_2 generation in the strain BR6. Mouse BR4 was mated firstly with a brother to start a strain designated BR4 (pink label), and secondly with an unrelated BR F_1 male, No. 1, to start the strain BR4 (blue label). Male No. 1 also sired the tumour-free female to start the strain BR16. Fig. 1 shows the provenance of the four strains. Each strain was continued by brother-sister matings. The F_2 females bred normally and raised their litters. Most of the F_3 females raised one litter and many raised two or three litters; subsequently they were subjected to forced breeding. Selected F_4 females raised one or more litters to continue the strains, but most F_4 females were subjected to forced breeding throughout their reproductive lives. All the mice were similarly housed and fed.

Pedigrees of all mice were kept and the record included a note of the coat colour; the BR F_1 hybrids were all of brown or "wild" colour and their progeny were classified as "wild," "white" or "black," minor variations being ignored. Selective breeding in the F_4 generation established "wild," "white" and

"black" sublines which, with rare exceptions, have bred true to type as far as the F_8 generation.

The results for the first three generations (hybrid generations F_2–F_4) are given in Tables I–IV. The "effective total" is the number of mice which survived to the age at which the earliest tumour was detected in any of the groups, namely 110 days in an F_4 mouse of strain BR4 (blue label). The "average tumour age" is the average age at which a tumour was first detected, the mice being inspected twice weekly. The mice alive with tumours include mice with nodules growing and regressing in repeated cycles; as described by Foulds (1949*b*), mice with these nodules almost always die with large typical tumours. Mice in which nodules were present at some period but not at the

TABLE I.—*Strain BR4 (Pink Label).*

Hybrid generation.	Effective total.	Tumour-free.				Tumour-bearing.				Total tumours.	Tumour incidence (%).	Average tumour age (weeks).
		Dead.		Alive.		Dead.		Alive.				
		No.	Average age at death (weeks).	No.	Average age (weeks).	No.	Average age at death (weeks).	No.	Average age (weeks).			
F_2	4	1	28	0	—	3	62	0	—	3	75	62
F_3	37	15	34	0	—	22	48	0	—	22	59	36
F_4	109	19	30	9	69	79	47	2	60	81	74	36

TABLE II.—*Strain BR4 (Blue Label).*

Hybrid generation.	Effective total.	Tumour-free.				Tumour-bearing.				Total tumours.	Tumour incidence (%).	Average tumour age (weeks).
		Dead.		Alive.		Dead.		Alive.				
		No.	Average age at death (weeks).	No.	Average age (weeks).	No.	Average age at death (weeks).	No.	Average age (weeks).			
F_2	6	2	42	0	—	4	61	0	—	4	67	51
F_3	52	13	33	4	70	33	59	2	69	35	67	35
F_4	101	4	38	19	60	69	47	9	65	78	77	35

TABLE III.—*Strain BR6.*

Hybrid generation.	Effective total.	Tumour-free.				Tumour-bearing.				Total tumours.	Tumour incidence (%).	Average tumour age (weeks).
		Dead.		Alive.		Dead.		Alive.				
		No.	Average age at death (weeks).	No.	Average age (weeks).	No.	Average age at death (weeks).	No.	Average age (weeks).			
F_2	4	2	34	0	—	2	58	0	—	2	50	50
F_3	34	8	35	1	55	23	53	2	56	25	74	39
F_4	98	11	30	6	59	75	50	6	58	81	83	35

TABLE IV.—*Strain BR16.*

Hybrid generation.	Effective total.	Tumour-free.				Tumour-bearing.				Total tumours.	Tumour incidence (%).	Average tumour age (weeks).
		Dead.		Alive.		Dead.		Alive.				
		No.	Average age at death (weeks).	No.	Average age (weeks).	No.	Average age at death (weeks).	No.	Average age (weeks).			
F_2	10	2	54	8	89	0	—	0	—	0	0	—
F_3	97	14	43	80	66	2	68	1	79	3	3	67
F_4	136	14	26	120	62	1	54	1	69	2	1	59

time of death are recorded as tumour-free. Many of the mice recorded as dead without tumour succumbed to epidemic infection about a year after the beginning of the experiment.

Tables I–III show that the incidence of tumours in the strains BR4 (pink label), BR4 (blue label), and BR6 was unequivocally "high." The three strains were substantially alike. Tumours developed, indiscriminately as it seemed, in "wild," "white," and "black" mice in the F_2–F_4 generations and continue to develop in "white" and "black" sublines up to the F_6 generation. The later age-incidence of tumours in the F_2 females was probably attributable to the breeding history; the F_2 females, in contrast with later generations, were not subjected to forced breeding.

The incidence of tumours in the BR16 strain (Table IV) so far is extremely low. The great majority of the mice survive and more tumours may yet develop, but the average age is already higher than the average age of tumour development in the other three strains. Whatever the final incidence, the contrast between strain BR16 and strains BR4 (pink label), BR4 (blue label), and BR6 is unmistakable.

Mammary tumour agent in transplanted BR1 tumours.

Extracts of transplanted tumours of the hormone-dependent BR1 strain (CR1 in the previous report by Foulds (1947)) were injected into female BA F_1 mice (C57 black ♀ × A ♂). According to previous experience in this laboratory (Gye, 1948), BA F_1 hybrids do not develop mammary tumours spontaneously although other laboratories record a low incidence; mammary tumours develop when tumour agent is supplied by foster-nursing or by injection.

The BA F_1 hybrids were 33–46 days old at the beginning of the experiment. Litter mates were allotted as equally as possible to three groups; one group received extracts of BR1 tumours; another group received extracts of spontaneous mammary tumours from mice of high-tumour strains; the third group received no injections. Tumours were ground in a mortar with sand and saline to make suspensions which ranged in strength, in different experiments, from 13 to 20 per cent W/V. The debris was removed by spinning in an ordinary centrifuge, and the supernatant fluid was injected, at weekly intervals, into the dorsal subcutaneous tissues, each dose being 1·0 c.c. Sixteen mice received each 4 injections of extracts of BR1 tumours of the first or third transplanted generations, five received 3 injections and two received 2 injections. Twelve mice received each 1 injection of an extract of a primary spontaneous C_3H tumour and 3 injections of primary spontaneous R3 tumours, and seven mice received 3 injections of the R3 extracts only. All the extracts were similarly prepared. The females were caged with males and subjected to forced breeding, litters being removed within 48 hours (usually within 24 hours) of birth.

Table V summarizes the results at 89 weeks from the beginning of the experiment. The "effective totals" comprise the mice which survived to the age when the first tumour was detected (180 days in a mouse injected with BR1 extract). Tumours developed with comparable frequency in the two groups which received tumour extracts and at similar times; none developed, within the period of observation, in the control mice, 11 of which survived, without apparent tumours at ages of 92–96 weeks. The extracts were not certainly free from living

cells, but the possibility that the tumours resulted from cell transplantation was negligible, being discounted by the development of tumours in mammary tissue at sites remote from the point of injection, by the long intervals between the injections and the appearance of tumours, and by the genetic differences between the mice providing the extracts and the mice which developed tumours.

TABLE V.—*Tumour Incidence in Injected and Normal BA F_1 Mice.*

Experiment.	Effective total.	Dead without tumour.		Alive without tumour.		Dead with tumour.	
		No.	Average age (weeks).	No.	Average age (weeks).	No.	Average tumour age (weeks).
BR1 tumour extract	21	2	55	0	—	19 (90·5%)	48
Spontaneous tumour extract	17	3	66	0	—	14 (82·4%)	43
Normal controls	12	1	82	11	94	0	—

The results indicated that transplanted BR1 tumours contained mammary tumour agent comparable in activity with that in spontaneous growths in high mammary tumour strains.

Spontaneous mammary tumours in RB F_1 mice.

A colony of RB F_1 mice (R3 ♀ × C57 black ♂) was maintained to compare the tumour incidence with that in BR F_1 hybrids. The mice were subjected to forced breeding like the BR mice and similarly housed and fed. Of the effective total of 36 females, 33 (91·7 per cent) developed mammary tumours. The average number of tumours per mouse was 2·5, 24 mice having from 2 to 6 tumours each. The average age at the first detection of a tumour was 25 weeks; the earliest tumour was recorded at the age of 17 weeks, and the latest at 54 weeks.

The 3 mice without tumours are alive at ages of 81, 81 and 85 weeks respectively. It is noteworthy that although continuously with males they have had no litters for about a year; previously litters were not recorded. No observations were made on virgin mice.

Incidence of tumours in (C57 black ♀ × agent-free R3 ♂) F_1 hybrids.

Female mice taken at random from the laboratory stock of C57 black were mated with males from a colony of R3 mice free from milk agent. I am indebted for the male mice to my colleague Dr. B. D. Pullinger, who raised the colony from a litter of R3 mice removed from their mothers immediately after birth and suckled by C57 black foster-mothers. Up to the present only 2 atypical mammary tumours, of squamous type, have developed at the age of 23 months amongst 117 breeding females over 9 months old (Pullinger, 1947, and personal communication). The F_1 hybrids were subjected to forced breeding throughout their reproductive lives.

One hundred female hybrids were reared; 21 died without tumours at an average age of 21 weeks and 79 are alive at an average age of 57 weeks. Of the surviving mice, 29 are less than a year old (average 39 weeks) and 50 are over a year old (average 67 weeks). The only tumour encountered so far was detected at the age of 64 weeks in a mouse which survives; the structure of the tumour

is unknown. Up to the present therefore the incidence in mice over a year old is 2 per cent.

Of the 23 tumours found in BR F_1 hybrids with normal R3 fathers, 20 were first seen in mice less than a year old, the average age at detection being 38 weeks. The incidence in BR F_1 hybrids with agent-free R3 fathers is much lower or much later.

Table VI summarizes for easy comparison the incidences of mammary tumours in the various hybrids.

TABLE VI.—*Summary of Tumour Incidences.*

		Effective total.	Tumour incidence (%).	Average tumour age (weeks).
BR F_1		71	15	40
BR F_1 (Agent-free ♂)		50	2	64
RB F_1		36	92	25
Strain BR4 (pink label)	F_2	14	64	55
„ BR4 (blue „)	F_3	123	67	39
„ BR6	F_4	308	77	35
Strain BR16	F_2	10	0	—
	F_3	97	3	67
	F_4	136	1	59

DISCUSSION.

Andervont (1945*a*) reviewed the records of relatively high incidence of mammary tumours in hybrid mice having low-tumour strain mothers, and hence presumed deficient in the mammary tumour agent. The possible explanations of the phenomenon were speculative because tests for the presence of tumour agent in the hybrids were not made. Subsequently, Andervont (1945*b*) and Andervont and Dunn (1948, *a*, *b*, *c*) investigated the occurrence of tumours in mice with strain C (low-tumour) mothers and strain C3H (high-tumour) fathers. Their results, which corresponded in some respects with my observations on hybrids with C57 black (low-tumour) mothers and R3 (high-tumour) fathers but differed in other respects, are discussed in later paragraphs.

The incidence of tumours in BR F_1 hybrids (C57 black ♀ × R3 ♂) resembled that recorded by others for various hybrids with low-tumour strain mothers and high-tumour strain fathers. Although the incidence was not high the tumours appeared at an average age of 38 weeks, which was within the range of age incidences of tumours in high-tumour strains. The histological structure was unremarkable except in two tumours which developed near the sites of injection of a carcinogen and in one tumour in a virgin. The sex-limited transplantation described in a previous paper was not peculiar to BR F_1 hybrids; as recorded in the paper which follows (Foulds, 1949*a*), some tumours in the reciprocal RB F_1 hybrids, which had a high incidence of tumours, behaved similarly. Mammary tumour agent, comparable in activity with the agent present in spontaneous tumours of high-tumour strain mice, was demonstrated in a transplanted BR F_1 tumour. The BR F_1 tumours, therefore, differed consistently in no particular from the typical mammary tumours of mice of inbred high-tumour strains. The tumours, however, were concentrated in a minority of litters of BR F_1 mice, the majority of families having none so that the total incidence was relatively low. Observations on the F_2 and subsequent

generations confirmed the implication that the BR F_1 hybrids were heterogeneous.

The descendants of the two tumour-bearing BR F_1 females had a high incidence of tumours at a relatively early age, and resembled in all respects mice of inbred high-tumour strains. The high incidence was maintained at least to the F_5 or F_6 generations. The diversity of genetic constitution in the F_2 and subsequent generations did not noticeably affect the incidence of tumours which developed indiscriminately in "black," "white" and "wild" mice and in each of the "black," "white" and "wild" sublines established by selective breeding. By contrast the tumour incidence in the progeny of a tumour-free BR F_1 female sired by a male that sired, also, one of the high tumour strains was extremely low or extremely late. These results recalled Bittner's (1940) observations on strain A mice nursed by C57 black foster-mothers. A minority (7·9 per cent) of the fostered A females developed mammary tumours, presumably because they received tumour agent from their strain A mothers before being transferred to foster-mothers, and the tumour incidence in their progeny was high (58·5 per cent). The majority of the fostered A mice developed no tumours and the incidence in their progeny was low (6·1 per cent).

In sum, the observations indicated that a minority of (C57 black ♀ × R3 ♂) F_1 hybrid females carried and transmitted the mammary tumour agent and developed mammary tumours, whereas the majority of the mice did not carry or transmit the agent and did not develop tumours. Direct and indirect evidence for the presence of tumour agent in tumour-bearing hybrids limits discussion to the source of the agent and the reason for its uneven distribution amongst the F_1 hybrids.

The sublines of C57 black mice maintained in various laboratories are not now identical and some possibly carry the mammary tumour agent, perhaps in attenuated or masked form. In this laboratory mammary tumours have not been recorded in the pedigreed stock although they have occurred, rarely, in unpedigreed mice derived from the C57 black stock as, for example, by random interbreeding; our subline maintains therefore the essential characteristics of the original stock. Mammary tumours have developed in two types of hybrid with C57 black mothers, namely (C57 black × R3) F_1 and (C57 black × C3H) F_1 but not in a third type (C57 black × A) F_1 (Gye, 1948, and this paper). Evidently the strain of the father had a substantial effect on the incidence of tumours in F_1 hybrids. The paternal contribution was not probably genetic for, as shown in this paper and in previous reports from this laboratory (Dmochowski, 1944, 1945), the C57 black × A hybrids developed tumours abundantly when tumour agent was supplied to them artificially. The uncompleted observations on hybrids having fathers belonging to a subline of R3 mice deprived of tumour agent accorded with similar observations by Andervont and Dunn (1948*a*, *b*, *c*) in showing a substantial reduction in the incidence of tumours in comparison with similar hybrids whose fathers derived from a line carrying the tumour agent. The experiment was open to the criticism, which Andervont and Dunn applied to their own experiment, that the agent-free and agent-bearing males were separated by several generations of inbreeding and possibly differed genetically. With this reservation, both experiments indicated a transmission of tumour agent by males of high-tumour strains to their hybrid offspring. The probability of this interpretation was increased by Andervont

and Dunn's (1948*a*) demonstration of tumour agent in the seminal vesicles of male mice of high mammary tumour strains. Genetically, the BR F_1 hybrids were uniformly susceptible to the development of mammary tumours, as shown by the high incidence which resulted from artificial administration of tumour agent (Dmochowski, 1946), and by the high natural incidence now recorded in the reciprocal RB F_1 hybrids. The relatively low natural incidence of tumours in BR F_1 was attributed provisionally, therefore, to the erratic transmission of tumour agent by male parents to their hybrid offspring.

Andervont (1945*b*) and Andervont and Dunn (1948*a*, *b*, *c*) studied the hybrid offspring of females of the strain C, having a mammary tumour incidence of less than 1 per cent, and males of the high-tumour strain C3H. Tumour agent was not demonstrable in the tumours which developed in the hybrids nor in lactating mammary glands of strain C females. No evidence was found, in any experiment, of transmission of tumour agent by strain C females. Tumour agent was demonstrated, however, in the seminal vesicles of male mice of high-tumour strains, and the incidence of tumours in (C ♀ × C3H ♂) F_1 hybrids was greatly reduced when the male present derived from a line of C3H mice lacking the tumour agent.

The C × C3H hybrids apparently differed from the BR F_1 hybrids in several important particulars: the incidence of tumours was much higher in C × C3H hybrids (about 60 per cent) than in BR F_1 hybrids (about 15 per cent), yet the average age at the appearance of tumours was much later (about 22 months compared with about 10 months); the C × C3H tumours were not concentrated in particular families as were the BR tumours, and tumour agent was not demonstrable in the tumours. Andervont and Dunn's most recent publication (1948*c*), received after the present investigation was completed, showed, however, that a minority of tumours in C × C3H hybrids corresponded with the tumours now described in BR F_1 hybrids. In general the (C × C3H) F_1 hybrids had the characteristics already enumerated; they had a high incidence of tumours at a late average age and not concentrated in particular families. Tumour agent was not demonstrable in the hybrids or in their tumours, and the offspring of tumour-bearing mice, whether obtained by back-crossing or by brother-sister matings, rarely developed tumours. Two litters of (C × C3H) F_1 were exceptional in that tumours developed in sisters at unusually early ages (4–10 months). The backcross offspring of two tumour-bearing females of one litter all developed tumours at average ages of 7 and 9 months respectively. The 4 tumour-bearing females of the other litter were mated with a brother, and breeding was continued by brother-sister matings. A tumour incidence of 85–100 per cent at ages of 5–10 months was observed in each generation as far as F_6. The presence of tumour agent was demonstrated by using hybrids from each of these lines as foster-mothers for susceptible mice. Tests for agent in the tumours were not reported. Evidently the exceptional " early " tumours in (C × C3H) F_1 hybrids were the counterparts of the tumours now described in BR F_1 hybrids.

It is concluded that a minority of BR F_1 hybrids carry a tumour agent, derived probably from the male parent, and develop tumours at a relatively early age, whereas the majority of the hybrids receive no tumour agent and remain free from tumours. The heterogeneity of the F_1 hybrids is attributed, provisionally, to erratic transmission of tumour agent by the male parent. Apparently a smaller minority of the hybrids used by Andervont and Dunn

develop early tumours exactly comparable with the BR F_1 tumours, but tumours develop in a high proportion of the remaining mice about a year later. The late tumours in which tumour agent has not been demonstrated account for the exceptionally high tumour-incidence in these particular hybrids. The present investigations provide no clue to the nature of the late tumours. It is evident, however, that the (C × C3H) F_1 hybrids, like the BR F_1 hybrids, are heterogeneous.

It is noteworthy that experimental deprivation of tumour agent greatly reduces but does not completely suppress the development of mammary tumours. My observations add two examples, namely the line BR16 established by breeding from a tumour-free hybrid female, and the colony of F_1 hybrids derived from C57 black females and R3 males deprived of milk agent. The aetiology of these "residual" tumours, which possibly correspond with the late tumours in (C × C3H) F_1 hybrids, is obscure.

SUMMARY.

Typical mammary tumours developed in about 15 per cent of the breeding females of the first generation hybrids, C57 black ♀ × R3 ♂ at an average age of 40 weeks. The incidence of tumours in the reciprocal hybrids, R3 ♀ × C57 black ♂, was 92 per cent at an average age of 25 weeks.

Mammary tumour agent was demonstrated in a transplantable tumour which originated in a (C57 black ♀ × R3 ♂) F_1 mouse by injecting tumour extracts into (C57 black ♀ × A ♂) F_1 hybrids which, otherwise, did not develop mammary tumours. The progeny of two tumour-bearing (C57 black ♀ × R3 ♂) F_1 females had a high incidence of mammary tumours (about 70 per cent) maintained through at least 5 generations. By contrast the progeny of a tumour-free female had a low incidence (0–3 per cent) similarly maintained. It was concluded that the F_1 hybrids were heterogeneous; a minority, concentrated in certain litters, developed mammary tumours, carried the mammary tumour agent and transmitted the agent to their offspring, which had a high incidence of tumours; the majority did not develop tumours and did not transmit the tumour agent to their offspring, which, with rare exceptions, did not develop tumours. Since the incidence in (C57 black ♀ × R3 ♂) F_1 hybrids was reduced or delayed when the male parent derived from a line of R3 freed from tumour agent, the low or moderate incidence of mammary tumours in hybrid mice with low-tumour strain mothers and high-tumour strain fathers was attributed, provisionally, to the erratic transmission of tumour agent by the male parents.

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MAMMARY TUMOURS IN HYBRID MICE: HORMONE-RESPONSES OF TRANSPLANTED TUMOURS.

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THIS paper records the later course of transplantable tumours already described (Foulds, 1947), and adds observations on the transplantation of tumours from other hybrids and on secretion in transplanted tumours. It provides evidence that hormone-responsiveness of transplanted tumours is not correlated with absence of the milk agent, that hormones regulate the activities of some transplanted tumours, and that responsiveness to hormones changes during serial transplantation.

MATERIAL AND METHODS.

The earlier report described the origin and transplantation of tumours of F_1 mice of the cross C57 black ♀ × R3 ♂ (BR F_1), and an accompanying paper (Foulds, 1949*a*) recorded the incidence of tumours in the reciprocal hybrids RB F_1 (R3 ♀ × C57 black ♂), in BR hybrids of the F_2 to F_5 generations, and in BA F_1 hybrids (C57 black ♀ × A ♂) supplied artificially with milk agent. (For reasons given in the accompanying paper the designation of the C57 black ♀ × R3 ♂ hybrids has been changed from CR to BR. Tumours called CR1, CR2, etc., in the earlier report are here designated BR1, BR2, etc.)

Transplantation was carried out by the method used in the earlier experiments. The recipient mice were BR F_1 or RB F_1 hybrids, and each series included normal males as well as normal females or oestrogenized males or both. As a rule, transplantation was not continued beyond the second passage.

Transplantation of tumours of RB F_1 hybrids.

The RB F_1 mice had the same genetic constitution as the BR F_1 mice used previously, but were nursed by their mothers of the high mammary tumour strain R3.

Twenty-five mammary tumours originating in 11 RB F_1 females were used for transplantation. Transplantation of 3 tumours was unsuccessful. Seven tumours from 5 mice grew in female or in oestrogenized male hosts, but grew tardily or not at all, within an observation period of 3 months or longer, in normal males. Implants of 15 tumours from 11 mice grew without conspicuous difference in male and female hosts; 4 of them were retarded slightly or inconstantly in males.

Transplantation of tumours of other hybrids.

Tumours from hybrids other than BR F_1 and RB F_1 were not examined systematically. The limited observations showed that 4 out of 7 tumours of

BR F_3 hybrids were transplantable in female but not in male hosts, and that 1 of 4 tumours from BA F_1 hybrids was partially and inconstantly inhibited in male mice.

Changes in tumours during serial transplantation.

The tumour BR1 was described in detail in the earlier paper as the prototype of hormone-responsive transplanted tumours. Serial transplantation was

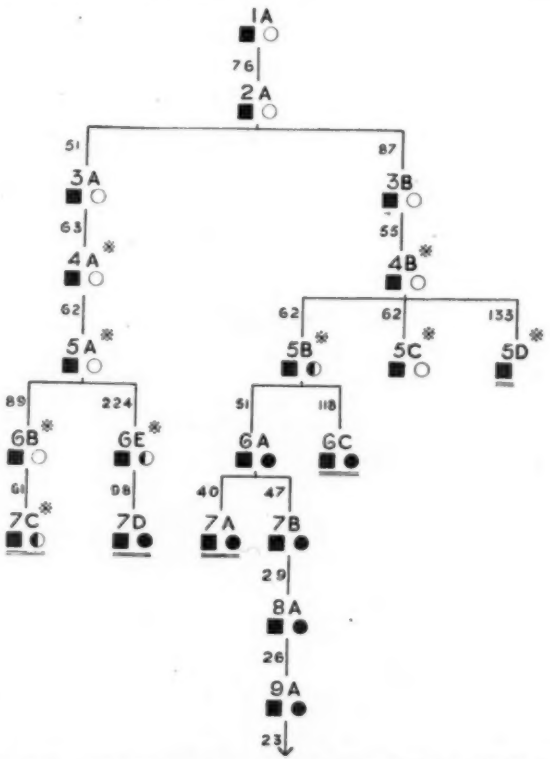

FIG. 1.—Transplantation of tumour BR1. Solid squares denote growth in females or oestrogenized males; solid circles growth in normal males and clear circles strong or complete inhibition of growth in males, less pronounced inhibition being represented by half-solid circles. The figures adjoining the vertical lines are the times, in days, between successive transplantations. Asterisks indicate the series in which milky secretion was conspicuous.

continued from 2 tumours of the second passage (Fig. 1). In one line of descent growth was consistently inhibited in male mice as far as the sixth passage. Two tumours of the sixth generation were transplanted about 12 months and 18 months respectively after the transplantation of the original BR1 tumour; implants of the first of these tumours grew after moderate delay in males as well as in females, and implants of the second grew equally in males and females.

Transplantation of this line was then stopped. Tumours in the other line of descent behaved similarly as far as the fourth passage. Three tumours of the fourth generation were transplanted. Implants of two of the tumours grew in female mice but not in males. The third tumour, transmitted by a large dose of minced tissue, grew in normal and in ovariectomized females and with only moderate delay in normal males. The tumour originated a distinct sub-strain of BR1 ; the tumours grew with equal facility in male and female hosts, they grew more rapidly than tumours of the original strain, they were less differentiated

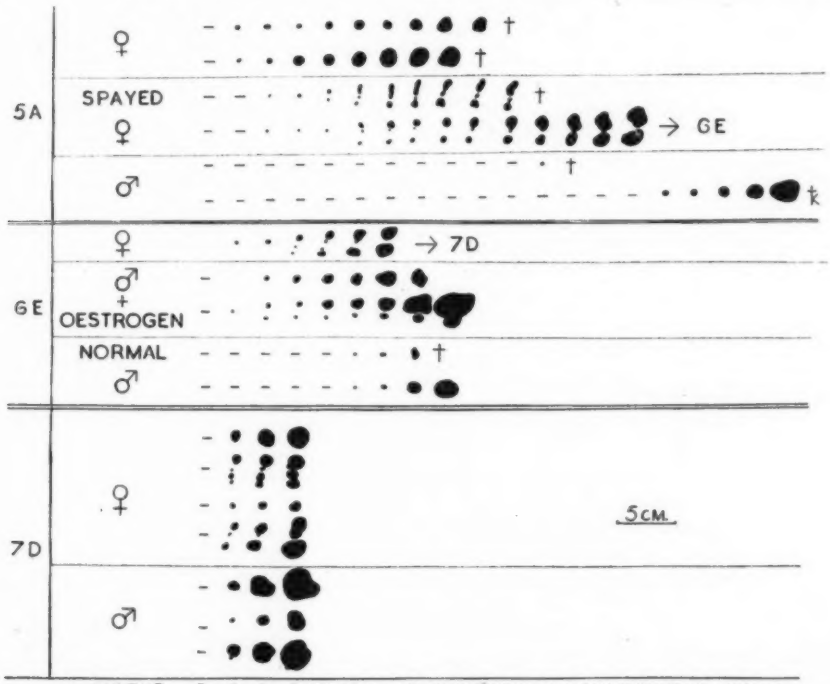

FIG. 2.—Three successive generations of tumour BR1 showing increase in rate of growth and loss of sex-difference.

histologically, and they were not milky in contrast with many tumours of earlier passages as described in the section which follows. These characteristics are maintained in the fifteenth and current passage. In the eleventh passage implants grew in each of 3 males but in neither of 2 sibling females. The anomalous result was not repeated and was unexplained. Fig. 2 illustrates the contrast between tumours of 3 successive passages. The intervals between successive transplantations as shown in Fig. 1 give a rough indication of the acceleration of growth in the subline that is being continued.

The earlier paper recorded transplantation of 2 tumours from mouse BR6. The second and larger tumour BR6B grew from the outset equally in males

and females, and maintained this behaviour up to the fifth generation, when transplantation was stopped. The earlier tumour was maintained in two lines of descent from the second generation onwards; the tumours of one line grew only in female hosts, whereas tumours of the other line grew also with inconspicuous delay in some males. The two lines were continued to the ninth and tenth generations respectively. In the later passages they were less clearly differentiated. In the eighth and ninth passages tumours of the first line grew in males as well as in females. Tumours of the second line continued to grow in males but, sometimes, only after long delay. The two lines thus converged without abrupt changes in behaviour.

One other BR tumour (BR4) grew only in females in the first three passages and in the fourth grew equally in males and females. One tumour from a BA F_1 hybrid behaved erratically during 4 transfers, after which transplantation was discontinued; in some series growth in females was superior to growth in males, but in other series no difference was apparent. The other transplantable tumours described in this and in the earlier paper were not carried through more than 2 or 3 passages.

Secretion in transplanted tumours.

In accordance with the general experience, histological examination disclosed varied degrees of secretory activity. Some BR1 tumours of the fourth to seventh transplanted generations, however, were conspicuously milky to gross inspection. The tumours were removed from mice in late stages of pregnancy, or from male mice carrying stilboestrol pellets; tumours from nursing females were not examined. They oozed milky fluid when cut; some contained small or large cysts with milky or creamy fluid; some discharged similar fluid through ulcerated patches of skin. When slices of tumours were fixed in formol saline the secretory tumours were evident to cursory inspection by reason of the milkiness of the fixative. Histological examination showed accumulations of secretion, patchy in distribution; tumour acini distended with secretion were contiguous with small, empty acini.

In the fifth passage of BR1, 23 male mice carrying stilboestrol pellets received implants. All the implants grew. Seven weeks after the transplantation the pellets were removed from nine mice. As previously recorded, removal of the pellets checked the growth of tumours temporarily, but no tumour retrogressed and soon all grew again at the customary rate. Three mice deprived of pellets died and were eaten by the survivors; the remaining 6 mice were killed 7 to 12 weeks after removal of the pellets. None of the tumours was milky. Mice which retained their pellets were killed during the same period. Tumours from 12 out of 14 mice were milky in greater or less degree; in one of the two mice with non-milky tumours the stilboestrol pellet was not found post mortem and evidence of oestrogenic action on the sex organs was lacking. Secretion was thus dependent on hormonal stimulation, whereas at that time growth was not so dependent.

Fig. 1 indicates (by asterisks) the passages in which conspicuously milky tumours were found. Examples were possibly overlooked or unrecorded in the first three passages. Thereafter milkiness was evident in pregnant females or in oestrogenized males in the series in which growth was inhibited in normal males.

None of the tumours in the later generations, when growth was rapid in normal males as well as in females, was milky, nor was any tumour of the other transplantable BR F_1 strains. Conspicuous milkiness was seen in the first passage of one BR F_3 tumour in oestrogenized males, and in one tumour from a mouse killed in late pregnancy in the second passage of a BA F_1 tumour. Two growths in the first passage of a RB F_1 tumour (BR10) were milky. Other transplanted RB tumours were not milky. Milky secretion evident to the naked eye occurred, therefore, in only a minority of those tumours whose transplantation was conditioned by hormones, and only under the stimulus of pregnancy or of an artificial oestrogen.

Response of transplanted tumours to pregnancy.

The transplanted tumours of two RB strains (RB9 and RB10) and one BR F_4 strain (BR F_4 90) which grew only in females increased in size whilst their hosts were pregnant and diminished after parturition. The regression was considerable but not complete (Fig. 3). The effect persisted in the second passage

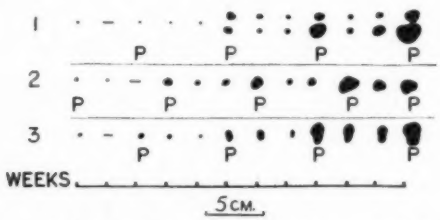

FIG. 3.—Three tumours selected from first and second transplanted generations of RB10. Pregnancy recorded at the weekly chartings marked "P."

of RB 10, which was not further transplanted, and in the second and third (current) passage of RB9. This pregnancy response was not observed in other transplanted BR or RB tumours, including those which secreted during pregnancy.

DISCUSSION.

At the time of the earlier report (Foulds, 1947) no information about the presence of mammary tumour agent or "milk agent" in BR F_1 mice and their tumours was available, but subsequent observations (Foulds, 1949*a*) provided evidence of the presence of tumour agent in transplanted tumours of the BR1 strain and in the BR F_1 mice from which derived two other tumours (BR4 and BR6) whose transplantation was conditioned by the sex of the host. It is now shown that mice of the reciprocal cross RB F_1 which receive tumour agent from their high mammary tumour strain mothers develop mammary tumours whose transplantation is similarly conditioned. It is evident that the dependence of some transplantable mammary tumours on appropriate hormonal conditions is not correlated with a deficiency of the milk agent.

The earlier report attributed the inhibition of implants in normal male hosts to lack of an essential stimulus which was provided by normal females and by oestrogenized males; it discounted the operation of the well-known resistance

to transplantable tumours in mice of mixed ancestry. The new observations support that conclusion by demonstrating two responses of established transplants to hormonal variations in the host. Pregnancy in females and artificially administered oestrogen in males elicits gross milky secretion in some transplanted tumours. Eisen's observations (1941) show similarly a pronounced though patchy secretion, in transplantable mammary carcinoma in rats, in response to oestrogenic stimulation above physiological levels, although transplantation was equally successful in male and female hosts. Withdrawal of oestrogen from male mice stops the secretion but growth continues after only a temporary check. Secretion in response to hormones is a property independent of, or additional to, the dependence of the tumours upon hormones for their establishment in new hosts. The acceleration of growth in two transplantable strains during pregnancy is another specific response; the growth of transplantable tumours in general is either unchanged or inhibited during pregnancy. The two responses, by secretion and by accelerated growth, are independent of each other, and each is manifest in only a minority of those transplantable tumours whose growth is inhibited in male hosts.

The inhibition of implants in male hosts is sometimes partial or doubtful at the first transplantation or after several transfers. Wider search would probably disclose still greater variety in the type and degree of response to hormones, and many different combinations of the relatively independent responses exemplified in the establishment of implants and in the growth and secretory activity of established tumours. The properties under discussion, however, are not immutable. This paper records new examples of changes in the course of serial transplantation. Most notably, tumour BR1, whose growth at first was consistently inhibited in male hosts, lost this characteristic and grew equally in male and female hosts; concurrently its growth accelerated, it became less differentiated in structure, and milky secretion ceased. Two other tumour strains changed similarly under observation. There is an evident tendency for hormone-responsive tumours to lose their distinctive qualities; if sufficient opportunity is provided by repeated transplantations they attain the unresponsive state which characterizes the generality of transplantable mammary carcinomata in mice. The paper which follows (Foulds, 1949*b*) describes hormone-responses and progression to unresponsive behaviour in spontaneous mammary tumours growing in their original hosts. Progression towards an unresponsive state occurs often in the primary tumours *in situ* but sometimes only after repeated transplantation in new hosts.

SUMMARY.

Some of the mammary tumours developing in RB F_1 hybrid mice (R3 ♀ × C57 black ♂), like tumours of the reciprocal BR F_1 hybrids (C57 black ♀ × R3 ♂), were transplantable, at first, into female or oestrogenized male mice of similar genetic constitution but not into normal male mice. The hormone-dependence was not correlated with lack of milk agent.

Pregnancy in females and an artificial oestrogen in males elicited gross milky secretion in mammary tumours of some transplantable strains. Tumours of two strains grew during pregnancy and regressed partially after parturition. The two responses, by secretion and by accelerated growth, were independent

of each other, and each was manifest in only a minority of those transplanted tumours whose growth was inhibited in normal male mice.

Responsiveness to hormones was not permanent. Progression of the responsive tumours to an unresponsive stage occurred when sufficient opportunity was provided by serial transplantation.

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SURVIVAL OF THE MILK FACTOR IN A TRANSPLANTABLE BREAST TUMOUR IN MICE.

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THE part played by the milk factor or the mammary tumour agent as one of the inciting factors in the development of spontaneous breast cancer in certain inbred strains of mice is to a large extent known. However, the part played by the milk factor in the propagation and survival of breast tumour cells induced by the factor still requires elucidation.

Bittner, Evans and Green (1945) demonstrated that the milk factor can be recovered from transplanted tumour cells of a spontaneous breast tumour of a high-cancer strain after ten serial passages in mice, which themselves did not possess the factor. Andervont (1944), however, failed to detect the agent in transplanted cells of a spontaneous mammary tumour of another subline of the same high-cancer strain even after the third serial transplant. In experiments previously reported (Dmochowski, 1948) the milk factor was not demonstrated in a so-called 63Ca transplantable breast tumour which had been transplanted for over 400 generations. It was not possible to state whether the milk factor was originally absent in the primary tumour, or whether it had been lost during the course of so many serial transplants.

In connection with these findings and the availability of a transplantable breast tumour originally induced in a C57 black low-breast-cancer strain female mouse by foster nursing by an R III high-breast-cancer strain female, experiments were carried out in order to ascertain whether the agent could be detected in this transplanted breast tumour after 42 serial transplants in C57 black low-cancer strain mice which do not possess the agent.

METHODS.

C57 transplanted breast tumour tissue, desiccated in the usual way (Dmochowski, 1946), was used as the source of material. This tumour induced in aC57 black low-breast-cancer strain female mouse by foster nursing by an R III high-cancer strain female, and designated as C57X breast tumour, was transplanted for 42 serial transplants in C57 black low-cancer strain mice. Before

use, the desiccated tumour tissue of the 42nd transplant was resuspended in distilled water in a proportion of 1 : 10, and injected subcutaneously every second day in 0·5 c.c. quantities into the mice. Each injection contained an amount of dried tissue equal to 0·05 g. of fresh breast tumour tissue. Each mouse received 15 injections, corresponding to a total of 0·75 g. of fresh breast tumour tissue.

C57 × R III hybrid mice, born and raised in the laboratory, were employed as test mice. The hybrid females of each litter, when four to six weeks of age, were marked individually and divided into experimental and control mice. All females were forcibly bred by removing the first three litters within 24 hours after birth, and then allowed to breed in a normal way and kept under similar conditions on a diet of " rat-cake " cubes and oats, with an unlimited supply of tap water.

RESULTS.

The results of the experiment are summarized in Table I.

TABLE I.—*Induction of Breast Cancer in C57 × R III Hybrid Females by Administration of Dried C57X Breast Tumour Tissue of the 42nd Transplantation.*

Number of mice.	Age of mice in weeks when first injected.	Number of injections.	Number of mice dying before earliest tumour appearance.	Number of mice with tumours. (%).	Average tumour age in months.	Average age of mice without tumours in months.
18	4–6	15	3	4 out of 15 (26·6)	14·5	17·1

No tumours developed in any of the 15 control C57 × R III hybrid females. As can be seen from Table I, dried C57X breast tumour in its 42nd transplant induced breast tumours in susceptible test mice. It would have been interesting to have tested the original tumour, and to compare the results with the present findings. This, however, could not be done in view of the shortage of space and animals—an unavoidable obstacle in this type of experiment.

DISCUSSION.

In the present experiments a breast tumour induced in a C57 black low-breast-cancer strain mouse was transplanted in other mice of the same strain characterized by low susceptibility to breast cancer and lack of the mammary tumour agent or the milk factor. In spite of the unfavourable medium for the factor it could still be detected after 42 serial transplants. Bittner, Evans and Green (1945) reported similar results after ten serial transplants of a spontaneous tumour which had developed in a C3H high-cancer strain mouse, and was transplanted in C3H mice deprived of the milk factor but still susceptible to it. According to Bittner, Evans and Green (1945) the presence of the factor in the transplanted breast tumour indicates that the milk factor is continuously produced within the transplanted breast tumour cells. Bittner (1948) has recently demonstrated that the factor is present in the tumour cells after ten transplants of mammary cancer which developed in C57 black mice following foster nursing by high-cancer strain females. Although in the present experiments the milk factor was found to be still present in the cells of the 42nd transplant of the breast tumour originally induced in a C57 black strain female by the factor present in R III high-breast-cancer strain mice, it is not possible to state what part, if any, is played by the factor in the propagation of the trans-

planted tumour cells. The C57X tumour is now in its 65th transplant, and will have to be tested again in order to throw some light on the question whether the presence of the milk factor is a permanent feature of the transplanted tumour cells. Should the test then fail to detect the factor, the results would suggest that the tumour cells continued to multiply although the factor had disappeared from the cells.

While the C57X tumour grew successfully in mice of the C57 low-breast-cancer strain, although it lacks the milk factor, it failed to grow in R III high-breast-cancer strain mice from which the factor responsible for the development of the original tumour was derived. These results show that the transplantability of the tumour was not connected with the presence of the milk factor in the hosts (C57 black mice), and the tumours grew equally well in females and in males of the C57 strain. Bittner (1947) has recently reported similar results. The transplantable tumours used by Bittner grew equally well in mice of the strain in which the tumours had originally been induced, whether they lacked the milk factor or possessed the same or different factor from that which was present in the tumour. The present results have again confirmed the observations made by previous workers that the reaction of the host to the transplanted breast tumour cells is dependent on the genetic relationship between the host and the transplanted tumour cells, and is not dependent on the presence of the milk factor in the host, as shown in Bittner's (1947) and the present experiment. It is possible with the help of the milk factor or the mammary tumour agent to induce breast tumours in C57 black strain mice which have an entirely different genetic constitution from that of the R III strain mice from which the agent was obtained (Dmochowski, 1944). These tumours, however, will grow on transplantation only in mice of the same genetic constitution (C57 black low-breast-cancer strain) as that of the mice in which the tumours originally developed, in spite of the absence of the agent in the mice acting as hosts for the transplanted tumour tissue, but will not grow in mice (R III high-breast-cancer strain) from which the agent originated because of their different genetic constitution. The genetic constitution of the host, therefore, and not the agent is responsible for the successful transplantation of breast tumours induced by the agent.

SUMMARY.

The results obtained in the experiments show that the milk factor is present in the cells of the 42nd transplant of breast tumour, originally induced in a C57 black low-breast-cancer strain mouse after foster nursing by high-breast-cancer strain R III female. They also confirm observations made by other workers on transplanted tumours of different origin that the genetic constitution of the host is responsible for the successful transplantation of breast tumours induced by the milk factor, and not the presence or the absence of the factor in the host.

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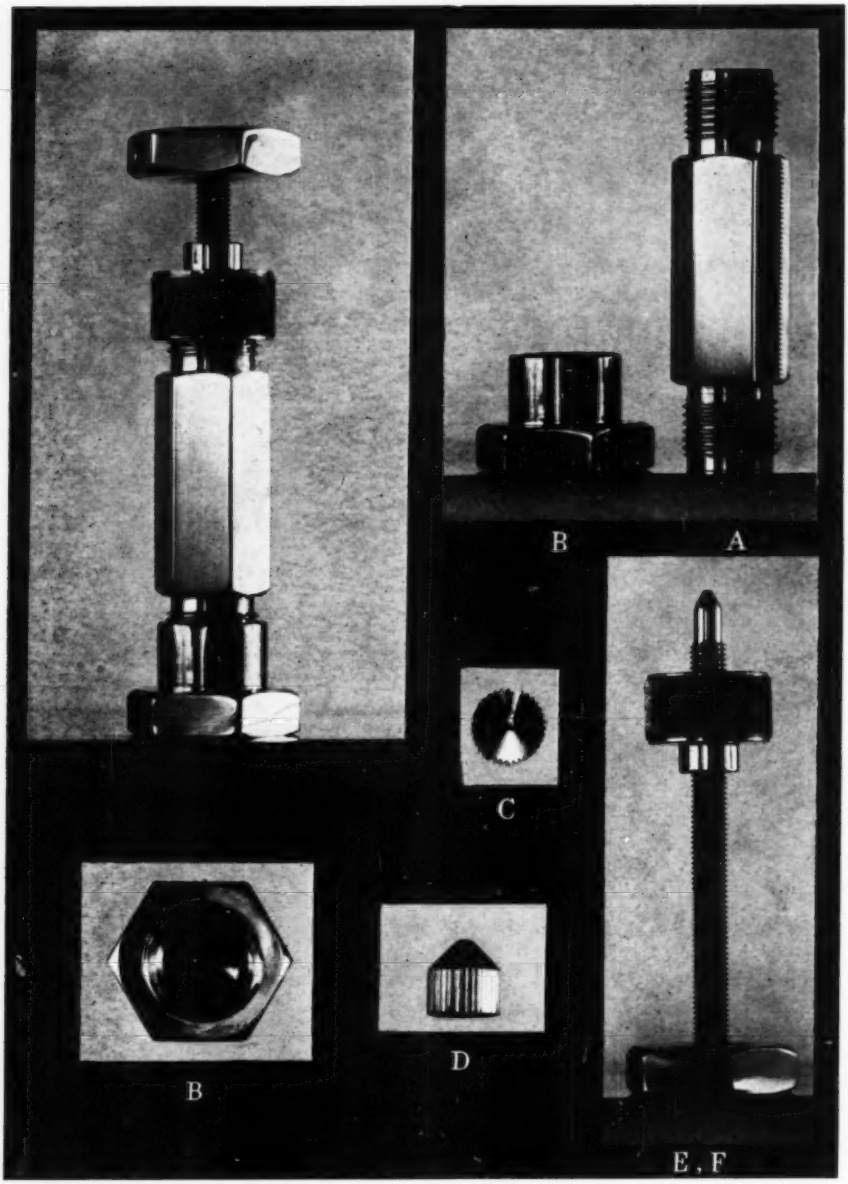

Fig. 1.—A pressure mincer for the preparation of tumour suspensions.

A PRESSURE MINCER FOR THE PREPARATION OF TUMOUR SUSPENSIONS.

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From the Laboratories of the Imperial Cancer Research Fund, N.W.7.

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THE pressure mincer described in this article was designed to facilitate the preparation of tumour suspensions for a quantitative study of the survival of tumours in the frozen state. The choice of principle and design was determined by the following requirements: (*a*) rapid reduction of tumour tissue to a suspension of fine fragments and separated cells, (*b*) ease of aseptic manipulation, (*c*) mechanical simplicity and (*d*) ease of cleaning.

The essential feature of this instrument is a grooved plunger which operates in a closed cylinder. The tissue is placed below the plunger, and on the application of pressure is forced through the grooves to the upper face.

The mincer and its component parts are shown in Fig. 1. All parts are machined from brass and are chromium plated.* It is important to have the plungers (C and D) fitted to the body tube (A) with the minimum clearance consistent with smooth working. The nose of each plunger fits accurately into the recess in the base plate (B). Similarly the nose of the fine plunger fits accurately into the base of the coarse plunger. The longitudinal grooves of the coarse plunger (C) are triangular in cross section, and are 1 mm. wide at the surface and 0·8 mm. deep. The grooves of the fine plunger (D) are 0·3 mm. wide and 0·3 mm. deep. The diameter of the body tube is 1·6 cm. Thus 1 mm. of displacement of the plunger corresponds to a volume of 0·2 ml. Cleaning is facilitated and design of the plungers is simplified by the provision of a detachable base plate. The operating screw (F) is of sufficient length to extrude the plungers from the lower end of the body tube.

The various parts of the mincer are sterilized separately in glass tubes. The only exceptions are the cap (E) and operating screw, the former being placed at a convenient level on the screw. The base plate is screwed tightly on the body tube just prior to loading with tumour tissue.

The maximum size of the pieces of tumour which should be used for mincing is determined by their consistency and the amount of fibrous tissue present. When possible it is preferable to avulse portions with fine, curved forceps from the tumour *in situ*, rather than cut it with knife or scissors after removal. If the tumour is not soft or friable the pieces used should not be larger than 0·7 mm. in diameter, i.e. 2/3rds of the length of the plunger grooves. When the pieces of tumour have been placed in the mincer, the plunger is inserted. This may be done by holding both the mincer and the glass tube containing the plunger in a

* It has been found preferable to machine the body tube and plungers from stainless steel in order to avoid the difficulties that may be encountered in obtaining an accurate fit with plated parts.

nearly horizontal position, approximating the open ends of each, and then raising the closed end of the glass tube. The fine plunger may be used alone for soft sarcomata, but other tumours require to be reduced first with the coarse plunger. After the plunger has been inserted the cap and operating screw are attached. The screw is turned until a definite increase in resistance is felt. This indicates that air has been expelled and the plunger is resting against packed tissue. At this point the distance between the surface of the cap and the underside of the head of the operating screw is measured on a special scale, which is graduated according to the volume below the plunger. The screw cap and operating screw are removed and a requisite volume of suspending fluid is introduced. The cap and screw are replaced, and the screw is turned until solid resistance indicates that all the reducible tissue has been forced through the grooves to the upper side of the plunger. The mincer is shaken immediately to disperse the mince in the suspending fluid. If the coarse plunger has been used the cap and operating screw are detached and the fine plunger is inserted. The cap and screw are replaced and mincing is completed.

The suspension may contain coarse fragments of fibrous tissue or portions of mince which have clotted before dispersal in the suspending fluid. These may be removed either by low speed centrifugation, or by filtering the suspension through a column of glass ballotini of suitable diameter.

The mincer, which is illustrated in Fig. 1, is the sixth of a series designed to operate with grooved plungers. All models have proved most satisfactory for the purpose for which they are intended, and it might be suggested that this type of mincer will, for special purposes, be suitable for preparing suspensions of mammalian tissue or fowl embryos infected with certain viruses or bacteria.

I wish to acknowledge my indebtedness to Mr. W. T. Caunt, senior technician, who has skilfully undertaken all necessary machine work in constructing the mincers.

A DRYING APPARATUS FOR THE STUDY OF TUMOUR TRANSMISSION.

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CERTAIN transplantable mouse tumours have been transmitted with dried tissue prepared with the apparatus to be described in this paper. The considerations which led to the development of the equipment and the results obtained with it are dealt with in another paper in this series. The present account is limited to a description of the apparatus and its use but, where necessary, a brief indication of the reasons for the choice of certain operating conditions will be given. The reader is referred to a report by Greaves (1946) for references and information about methods of drying biological materials from the frozen state, and no review of the subject will be attempted here.

Before the present apparatus was constructed a number of transplantable mouse tumours (R3 and C3H sarcomata) were dried with the equipment described by Knox (1939). Although Knox obtained highly active desiccates of filtrable fowl tumours, the results obtained by the writer with the mouse tumours were negative. However, this experience, combined with trials of technical modifications, served to clarify the problems of alternative design. In all, eleven lots of C3H sarcoma were dried and tested during the preliminary work. None of these lots of dried material produced tumours on inoculation into C3H mice, but the conditions, in one way or another, were suboptimal, and these negative results are to be contrasted with the positive results reported in another paper (Gye, Begg, Mann and Craigie, 1949).

The apparatus described in this paper is designed to dry small, single samples of tumour suspension rapidly at a temperature not exceeding a predetermined maximum, the purpose of drying being to study the conditions favouring survival of tumour activity. It was considered that the freezing points of the eutectic mixtures of water and the inorganic salts present in the suspending fluid or within the cells could not be disregarded as potentially important factors. The freezing points and percentage concentration of the eutectic mixtures of NaCl, KCl and $CaCl_2$ are given in Table I.

TABLE I.

Salt.	Eutectic mixture with water. Salt. (%)	Freezing point. (°C.)
NaCl	30·4	— 21·2
KCl	25·0	— 10·7
$CaCl_2$	48·0	— 52·0

If cells are suspended in 0·85 per cent NaCl and the suspension frozen at —20°C. it is to be expected that a deleterious concentration of NaCl will accumulate between the cells and the ice crystals. This view is supported by the results of comparative tests in which suspensions of C3H sarcoma mince in saline and Ringer solution were held in the frozen state at — 20° C. and — 79° C. for several hours. A further question arises in connection with the physical events within the cells during freezing and drying. If the water within the cell is frozen, it is to be expected on theoretical grounds that temperatures below the freezing points of the eutectic mixtures of the inorganic salts would favour survival of activity. So far no attempts have been made to dry tumour tissue from temperatures below — 52° C. (Table I), and investigations have been confined to the range between — 22° C. and — 30° C.

The problem has been simplified to some extent by the finding (Craigie, 1949) that dextrose favours the survival of tumour activity on freezing and thawing. Thus electrolytes can, with advantage, be omitted from the fluid used for suspending tumour mince in drying experiments. There is, however, an equally important reason for the use of dextrose suspending fluid. Tissue mince suspended in saline or Ringer solution and frozen in a thin layer progressively separates from glass during drying. Consequently the drying process becomes greatly impeded because of the low rate at which thermal energy is conducted across the intervening vacuum, while dried fragments may break off and may be

swept across to the condensing flask by the current of water vapour. The addition of dextrose (5 per cent or more) to the tumour mince or suspension greatly increases its cohesiveness and its adhesiveness to glass. The thermal conductivity of the dried material is adequate for the rapid drying of thin layers.

Water is removed from the tumour suspension by the low temperature condensation method. Condensation at — 79° C. presents no difficulty, but there are technical problems in maintaining the drying tissue at a constant temperature level. If the lead from drying flask to condenser is of sufficient diameter and the vacuum is adequate, the problem is not one of cooling, but of supplying the maximum of thermal energy for rapid evaporation without heating the frozen mass above the chosen maximum temperature. It was considered undesirable to attempt to control the temperature of the drying mass on the basis of thermocouple readings. There is no guarantee that a thermocouple junction can be placed at that point in the frozen mass which will be the last to dry. On the contrary, it is to be expected that such an arrangement is an impossibility, if any significant amount of heat is conducted from the leads at room temperature to the junction. It was decided, therefore, to control the temperature by immersing the drying flask in a suitable bath, and to aid heat transfer by mechnical stirring.

It is not possible to state the actual temperature difference between the bath and the drying surfaces of the tumour suspension, but the fact that a vapour flow indicator does not indicate any further rise in evaporation rate when a certain speed of stirring of the bath has been reached, shows that the differential can be limited to that imposed by the thermal conductivities of glass and the dried mixture of dextrose and tissue.

It may also be noted that the conventional methods of following the course of drying by means of vacuum gauges, or high-frequency discharge, have been dispensed with. It was found possible to construct a vapour flow indicator of sufficient sensitivity to serve the need. This indicator is inserted in the outlet tube from the drying flask and will be described later in this paper. The elimination of connections for vacuum and temperature observation has simplified the design and operation of the apparatus.

APPARATUS.

The drying system.

This is constructed entirely of glass and is illustrated in Fig. 1. It is connected by a ground glass taper joint to a type 2 Speedivac vacuum pump (W. Edwards & Co., Ltd.), which has a displacement of 48 litres per minute and is capable of producing a vacuum of 0·00001 mm. Hg. It may be noted that the vapour pressure of ice at — 79° C. is 0·0004 mm. Hg. The glass system consists of five parts connected together by interchangeable ground glass taper joints, which are sealed with silicone high vacuum grease. Part 1 includes a two-way mercury seal tap giving connections (*a*) pump to system and (*b*) pump to atmosphere with the system sealed. The U-tube (2, Fig. 1) serves a double purpose as a trap for oil diffusing back from the pump, and as a water vapour trap should the temperature of the condenser (3) be allowed inadvertently to rise. The arm of the U-tube distal to the pump is extended horizontally and then downwards, to form the exit tube from the condenser flask. This exit tube is furnished with a ground glass taper which fits the neck of the flask. A short side arm and tap

attached to the condenser flask provide the means of breaking the vacuum. The inlet tube to the condenser (3) is jointed to a right-angled tube (4), in which a horizontal or vertical vapour flow indicator may be accommodated. The drying flask (5) is attached to tube (4).

The glass system is supported at the points marked in Fig. 1 by clamps sliding on pairs of vertical rods attached to the bench (see Fig. 2, *a*).

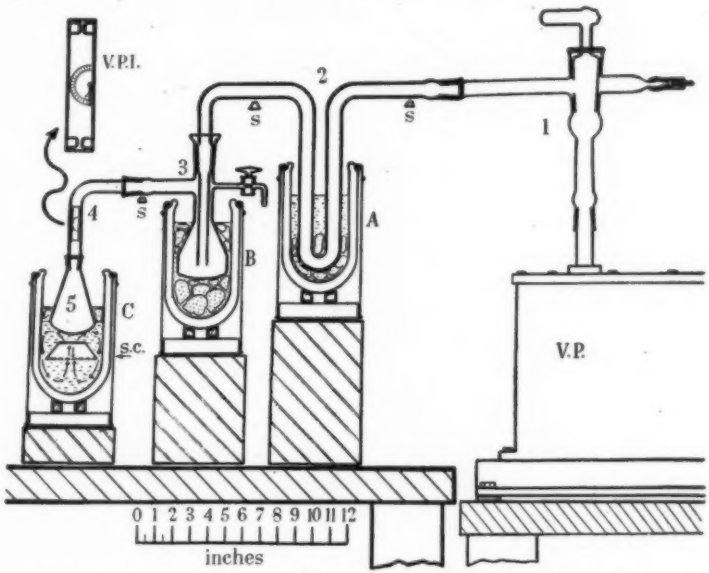


FIG. 1.—1, 2, 3, 4 and 5—parts of all-glass drying system. 3, Condenser flask ; 5, drying flask. *s*, Suspension points of drying system ; *s.c.*, stirrer cone. A, B and C, thermos jars for CO_2 ice-ethyl cellosolve baths. V.P.I., vapour flow indicator ; V.P., vacuum pump.

Accessory equipment.

(1) *Low temperature baths.*—Thermos jars are used to hold the mixtures of ethyl cellosolve and CO_2 ice required and their positions are shown in Fig. 1. Baths A and B are saturated with CO_2 ice, giving a temperature of $-79°$ C. CO_2 ice is added as required to bath C to maintain the desired temperature.

(2) *Stirring apparatus.*—The stirrer is a truncated hollow cone of copper attached to a vertical rod (Fig. 1 and 2, *b*). It moves vertically through a distance of 12 mm., producing an upward central current of fluid which impinges on the convex bottom of the drying flask. It is operated by means of the variable speed device shown in Fig. 2, *a*, *b*, *c*. The stirrer rod is attached by means of a slotted clamp which facilitates assembly with the thermos bath and drying flask. The rubber ring which drives the disc of the variable speed gear is mounted on a wheel which can be moved along the horizontal bar. This bar is mounted on two spring-loaded arms, and can be instantaneously released if it is desired to stop the stirrer. The variable speed gear is driven by shaft and pulley from the driving belt of the vacuum pump.

(3) *Vapour flow indicator.*—This consists of a light vane suspended freely from a pivot so that it can be displaced by a current of gas or vapour. Several indicators of this type have been made and tested. The one shown in Fig. 1 is mounted in the vertical limb of tube 4, which connects drying flask and condenser. A photographically reduced protractor scale is cemented to a piece of perspex 2·5 × 13 × 65 mm. and the pivot is centred at the origin of this scale. The vane is a piece of photographic dry mounting tissue 3 × 8 mm., which is attached by shellac to a piece of fine copper wire 0·01 mm. in diameter. The vane is mounted at an angle of 45° to the wire. The free end of the wire is bent back towards the pivot in a plane at right angles to the scale, thus serving as an anti-parallax device. The combined weight of vane and wire is 3·7 mg., so that the vertical component of its weight at a displacement of 1° from its zero position is 0·065 mg.

(4) *Vibration dampers.*—Owing to the design of the glass-drying system and the use of a sensitive vapour flow indicator, it is necessary to check the slight vibration arising from the electric motor driving the pump. The drying system is mounted separately from the pump unit, which is attached with rubber cushions to a small rigid bench. The motor was removed and mounted on rubber cushions interposed between it and the base plate to which the motor and pump are attached by the makers. The residual vibration was neutralized by interposing a thrust bar between the base of the motor and a point on the pump casing determined by experiment.

METHOD OF DRYING.

Preparation of tumour tissue.

The tumour is minced in the pressure mincer designed by Craigie (1949), and the mince is suspended in diluting fluid containing 5·3 per cent dextrose. The suspension is placed in a sterile drying flask and frozen in as even a layer as possible over the bottom of the flask. The distribution of the frozen mince is readily controlled if the level of ethyl cellosolve in the freezing bath is adjusted so that the flask will float with its neck resting on the rim of the thermos jar and can therefore be spun quickly by hand. The temperature of the freezing bath may be — 30° C. or lower. It has not yet been determined whether a preliminary reduction of temperature to — 79° C. for a short time favours or prejudices survival of activity on drying.

Drying.

The flask containing the frozen mince is attached to the drying system and the bath C with the stirrer is placed in position. Baths A and B are saturated with CO_2 ice, and when they have reached a temperature of — 79° C. and bath C has been adjusted to the desired temperature, the pump is started. The variable speed gear of the stirrer is quickly checked to make sure that maximum evaporation, as shown by the vapour flow indicator, is taking place. At this stage a low reading of the vapour flow indicator will provide evidence of a leaking joint. During drying the drying flask bath is cooled as required by the addition of small pieces of CO_2 ice and the reading of the vapour flow indicator is recorded at intervals. In the earlier stages of drying the bath requires little cooling, but when drying nears completion a more frequent addition of CO_2 ice becomes necessary. When drying is almost complete it is usually observed that vapour is liberated

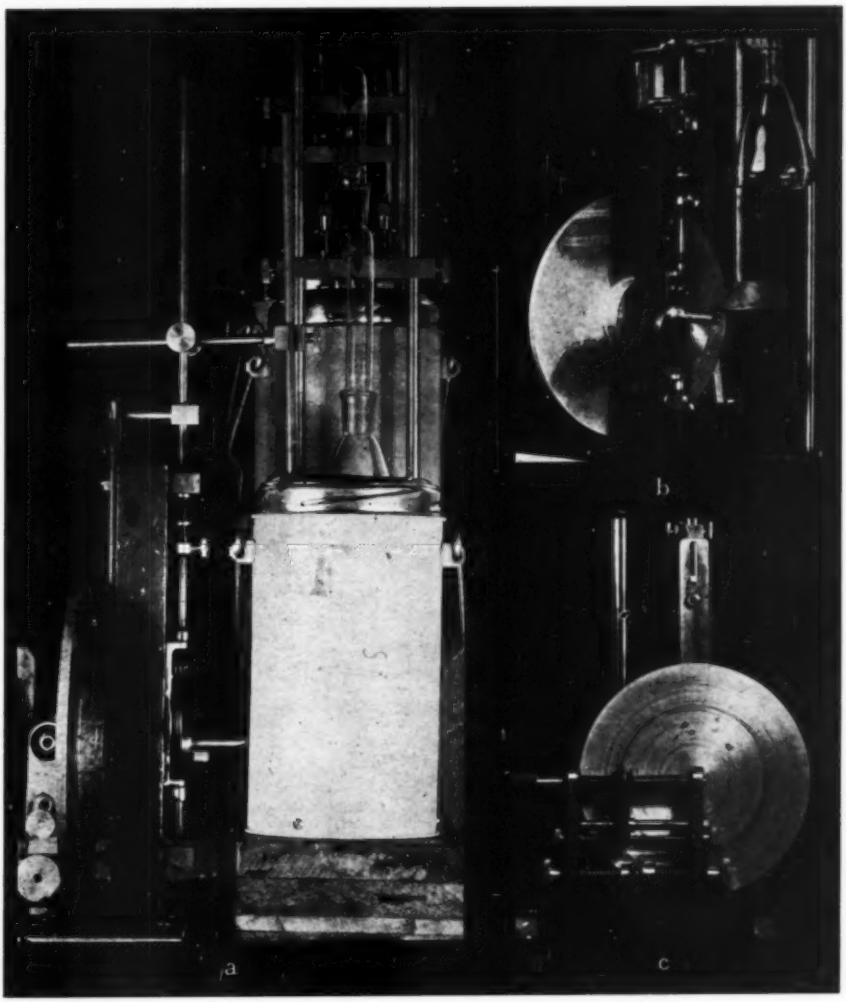
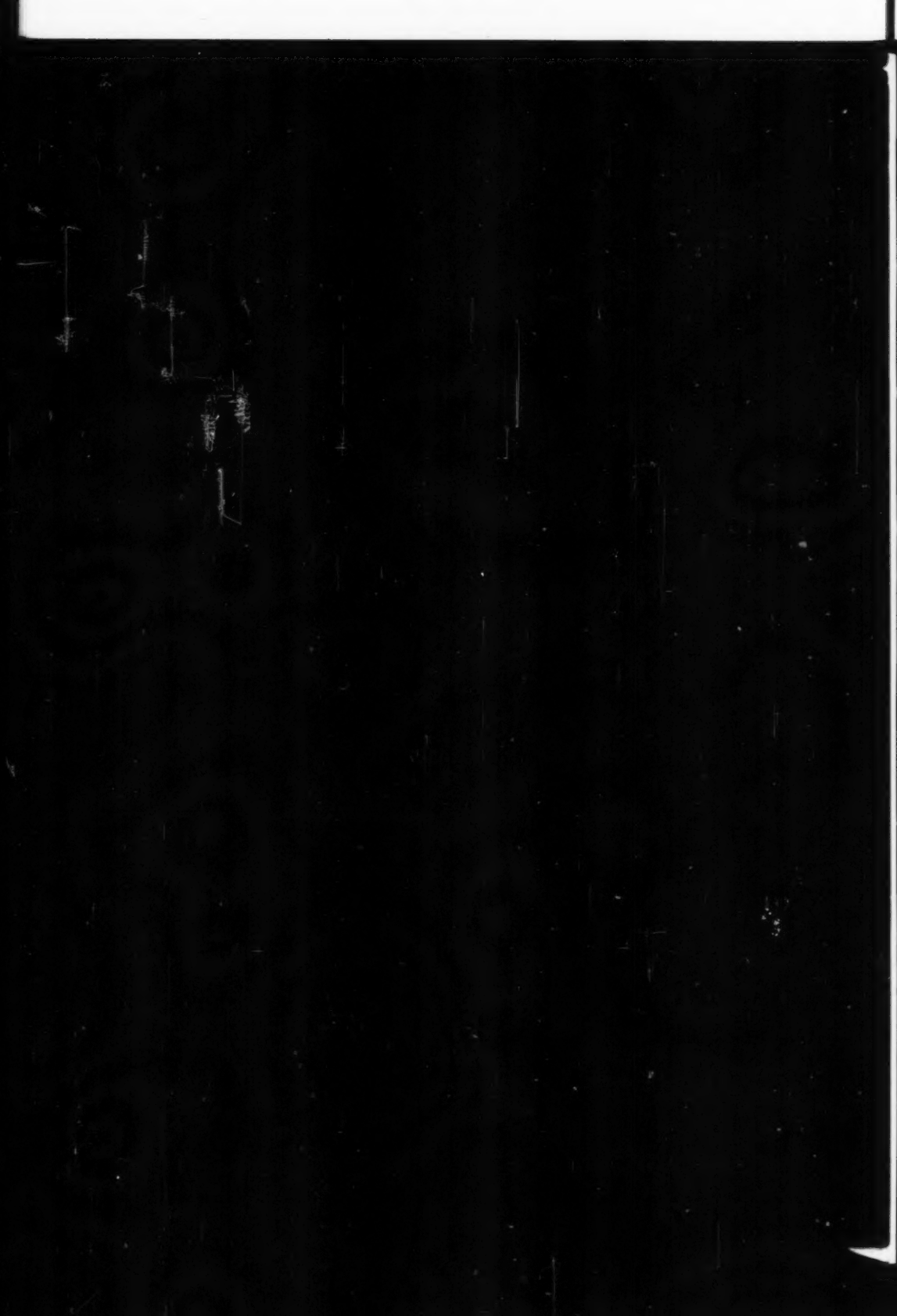


FIG. 2.—Stirring apparatus.



in small bursts, shown by irregular movements of the indicator between 3° and 10°. These bursts last for a few seconds, and are separated by slightly longer intervals. After the indicator has reached zero, as checked by inspection with a hand lens, pumping is continued for 30 minutes or some longer convenient interval.

Reconstitution of dried material.

After the vacuum has been broken and the drying flask detached, the material is reconstituted *in situ* by the addition of water to which other materials may be added. The fluid is carefully introduced into the flask at a point outside the area occupied by the dried material and, by slowly tilting the flask, is allowed to percolate the mass from below. Absorption is rapid, since air bells are not entrapped in the material. Mixing or shaking are avoided until the mass is completely wetted.

Acknowledgments are due to Mr. W. T. Caunt for the construction of the stirring apparatus, stands and motor mounting, and to Mr. C. J. Greenall, Glass Printers, Ltd., for his interest in the construction of the glass drying system.

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A STUDY OF CELL SURVIVAL IN EMBRYONIC TISSUE GRAFTS IN INBRED STRAINS OF MICE UNDER VARIOUS CONDITIONS.

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WHEN normal laboratory stock mice are grafted with embryo tissue mince the embryonic cells grow and divide for a short time, usually for a week or ten days, to form an embryoma which steadily retrogresses and finally disappears. The cells of the host and graft are antigenically unlike, and consequently those immunological reactions which dispose of foreign proteins come into play, kill and remove the implanted cells. In homozygous mice the cells of host and graft are alike and no such reaction occurs ; the cells which are grafted survive, grow and form large embryomas which persist for months or indefinitely.

The following series of experiments was undertaken to gain knowledge of the behaviour of such embryo grafts under various conditions. The results obtained were subsequently used for comparison in somewhat similar experiments in which tumour tissue was used.

Series I.

An attempt was made to estimate (*a*) the capacity for continued growth of embryo tissue implanted subcutaneously, and (*b*) the possibility of "passaging" teratomas so produced through a series of mice, in the way that tumours are passaged.

The mice used were from the C3H strain. The embryos were all approximately at the 8 mm. stage. The embryo tissue was finely minced with scissors, and diluted with normal saline sufficiently to enable it to be drawn easily into a Bashford syringe. The dose of tissue was in each case 0·05–0·1 c.c. and was inoculated into the right flank. 144 mice were used, divided into batches of 6 each. (No difference was observed between the behaviour of males and females.) In every mouse the graft took and teratomas appeared and persisted, increasing slowly in size. In 4 weeks the minute mass had grown to a lobulated swelling measuring approximately 10 × 5 mm. Growth continued thereafter very slowly, and the maximum size ever reached was 20 × 10 mm., attained in 24 weeks. Once the maximum size was reached the mass persisted indefinitely. The teratomas consisted of a mélange of normal tissues arranged in an irregular manner around cysts containing hair, skin and various secretions. Skin, cartilage, bone, marrow, muscle, fat, cubical and columnar epithelium, lymphoid tissue, blood vessels, glandular structures and pigment were all found. No necrotic patches were present, nor was there evidence of any reaction on the part of the host.

In order to discover the best time to transplant such a primary teratoma for passaging mice were killed once a week, beginning a week after inoculation, the teratoma removed, lightly minced with scissors and a small portion (approximately 0·03–0·05 c.c.) inoculated with a grafting needle into the right flank of six C3H mice. These formed the 1A generation. In this generation increase in size of the graft occurred in 66 per cent of transplants done before the 4th week, in 33 per cent or less in those between the 4th and the 8th week, while after the 10th week practically no increase in size took place, the small masses either persisting in practically the same state as when implanted, or actually disappearing. Several of those passaged at the first and second weeks were again divided and a portion transplanted. These only increased to between 12 × 6 mm. and 4 × 4 mm., and were left *in situ* for 11 weeks. This is the 2A generation. The largest was again passaged, giving the 3A generation, but no growth occurred this time, the nodule merely persisting, with its tissues in the adult stage of differentiation. A further attempt to passage a teratoma to the 2A generation was made with two 1A's transplanted at 18 and at 20 weeks. These resulted again in persistence only, no increase in size occurring.

Fig. 1 shows these results.

It is thus evident that embryonic tissue possesses a power to continue growth which lasts only a certain time and is not enhanced by repeated passages, unlike that of tumours.

Series II.

It was then decided to try the effect of subjecting the embryo tissue to various conditions before implanting.

A. *The effect of very fine mincing.*—In this experiment the C57 Black strain of mice was used. One group of 6 received embryo tissue minced with scissors

as in Series I. Teratomas appeared in ten days and reached a size of 20 × 10 mm. in 6 weeks. Another group of 6 mice received a similar dose of mince, but in this case the embryo tissue had been passed through a Craigie pressure mincer, and was so finely divided that it could be drawn up through a 26-gauge hypodermic needle. In these mice the teratomas did not begin to increase in size for 14 days, and in 6 weeks had only reached a size of 12 × 4 mm.

Fine mincing therefore delays and retards growth, but does not kill all the cells.

TERATOMA	1A. TRANSPLANT	2A. TRANSPLANT	3A. TRANSPLANT	TERATOMA	1A.	2A.
1 WEEK.	4+2− →	3+3− →	→	13 WEEKS	3+3− →	
2 WEEKS.	4+2− →	3+3− →		14 "	2+4− →	
3 "	4+2− →			15 "	2+4− →	
4 "	2+4− →			16 "	3+3− →	
5 "	3+3− →			17 "	3+3− →	
6 "	2+4− →			18 "	2+4− →	
7 "	2+4− →			19 "	1+5− →	1+1− →
8 "	2+4− →			20 "	2+4− →	
9 "	2+4− →			21 "	1+5− →	2+4− →
10 "	3+3− →			22 "	1+5− →	
11 "	3+3− →			23 "	0+6− —	
12 "	1+5− →			24 "	1+5− →	

FIG. 1.—Series I: Passage of teratomas.

B. *The effect of storing finely minced embryo tissue in glycerol at 4° C.*—The embryo tissue was passed through the Craigie pressure mincer and then stored in ampoules at 4° C. as follows:

2·5 c.c. glycerol.
0·5 ,, cystein hydrochloride buffered to pH 7·5—final dilution 1 in 500.
1 ,, normal saline.
1 ,, embryo mince.

After storage for one week the excess glycerol was pipetted off and the tissue inoculated into 6 mice. Small lumps were palpable at the site of inoculation for 3 weeks. All traces had disappeared by 4 weeks. No growth occurred. The cells were dead.

C. *The effect of diluting finely minced embryo with a cystein-dextrose solution.*—C3H mice were used. The embryos were pressure minced as above and diluted as follows :

7 c.c. of 5·3 per cent dextrose solution.
1 ,, of cystein HCl pH 7·5.
2 ,, embryo mince.

0·1 c.c. was injected into each of 6 adult mice. Five out of the six mice developed teratomas in the 5th week, but they were very small (average 5 × 2 mm.). The dextrose-cystein combination has a deleterious effect and markedly damages the cells.

D. *The effect of freezing finely minced embryo to* — 79° C.—Strong A mice were used. The embryos were pressure minced and diluted 1 in 1 with Ringer solution.

SERIES II		COARSE MINCING	FINE MINCING
A		6+	5+1-
B	GLYCEROL	—	
C	DEXTROSE	2+4-	3+3-
D	FREEZING TO −79°C FOR 24 HOURS.	—	
E	FREEZING IN DEXTROSE TO −79°C FOR 1 HOUR OR LONGER (UP TO 48 HOURS).	—	

FIG. 2.—Series II: Effect of subjecting embryo tissue to various conditions before implanting.

Six mice were injected as controls and all had teratomas. The rest of the material was frozen in ampoules to — 79° C. and stored. (In freezing the temperature was dropped 2·5° C. per minute to — 5° C., then rapidly to — 20° C., and the ampoule then stored in CO_2 snow.) After one week and after two weeks 6 mice were injected with the frozen material. No teratomas developed. Freezing to — 79° C. for one week kills the cells.

E. *The effect of freezing finely minced embryo tissue to* — 79°*C. in cystein-dextrose.*—C3H mice were used. The solution was the same as that used in C. The tissue was frozen for periods of 1 hour, 3 hours, 6 hours, 12 hours, 24 hours and 48 hours. No growth of tissue occurred in any except the unfrozen controls. The cells were killed, even in 1 hour. The experiment was repeated with C57 mice, omitting the cystein, and inoculating after 24 hours of freezing in dextrose. No growth of tissue occurred and the cells were dead.

Fig. 2 shows the results.

It therefore appears that fine mincing alone retards the growth of the teratomas, that dextrose and cystein severely damage the cells, and that freezing to — 79° C. with or without dextrose and cystein kills all the cells.

In the freezing experiments mice were killed daily and the implant sectioned. At 2 days (in material frozen 24 hours) a few surviving cartilage cells were found, but most of the tissue was dead. At 3 days there was a polymorph and macrophage reaction around the dead tissue, very little of which was recognizable. At 13 days only islands of dead cartilage surrounded by macrophages and giant cells could be seen.

Sections of tissue treated with glucose without freezing show healthy embryo tissue. Sections of the glycerol-treated tissue show remnants of cartilage and large numbers of host giant cells and macrophages.

CONCLUSIONS.

1. Coarse and fine embryo mince untreated will live and grow when implanted under the skin of mice of the same inbred strain.
2. Treatment with dextrose and cystein retards growth.
3. Freezing to — 79° C. for 1 to 24 hours with and without dextrose or cystein prevents all growth and kills all the cells.
4. Treatment with glycerol and storage at 4° C. kills the cells and prevents growth.

THE SURVIVAL OF ACTIVITY OF MOUSE SARCOMA TISSUE AFTER FREEZING AND DRYING.

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NUMEROUS authors, from Ehrlich (1907) to Breedis and Furth (1938), have reported that tumour tissue, both sarcomatous and carcinomatous, can be kept for long periods, certainly more than a year, at low temperatures from —10° C. to —79° C., without losing the power to start tumours afresh in the appropriate animals. From this and other papers in the present series it will be seen that we have amply confirmed these findings. Extremely active material capable of starting fresh tumours in less than two weeks has been obtained from both chemically induced and spontaneous sarcomas frozen at —79° C. for twelve months, and from sporadic carcinomas frozen at —79° C. for over three months. This phenomenon of survival of activity of cancer cells under conditions which are known to kill normal mammalian embryo cells (Mann, 1949) appears worthy of investigation. since it involves the question, first raised by Ehrlich in 1907, in connection with a possible bacterial source of cancer, of whether extreme cold can be used to destroy mammalian cells, and simultaneously to preserve any possible intra- or extra-cellular agent capable of starting a tumour *de novo* in cells of another host. If the continued activity of such refrigerated tissue is due to survival of cells of the original animal, then the tumour produced in the new host is merely a transplant. If, however, it can be shown that the cells in the refrigerated tissue are dead, then the new tumour must be produced by an agent (which would then be the " continuing cause " of cancer) which is a separate

entity from the cells in which it lives, and which is capable of surviving under certain conditions when they are dead. Such transmission would be "cell-free," and would place any tumour so produced in the class of virus tumours, among the known virus chicken tumours, the Shope papilloma of rabbits, Bittner's mammary carcinoma and Lucké's adenocarcinoma of the frog.

The proof that prolonged refrigeration kills mammalian tumour cells is difficult, and we are aware that in the past it has always been held, except by Cramer (1930), that the survival of only a few cells in the tumour mass accounts for the tumour in the new host.

We have investigated the problem from a variety of angles and have concluded, as will appear, that the cells are dead.

In the first place it must be realized that the sarcomas of mice which we have used are three well known to laboratory workers, one a methylcholanthrene sarcoma (C.48 in the inbred strain C57 Black), and the other two arising as stromal transformations in transplants of sporadic mammary cancers investigated by R. J. Ludford (the R3β R.T. and the C3H R.T.). These three tumours have hitherto resisted all attempts at cell-free transmission, so that our results, as will be seen, point, not to the accidental discovery of three new mammalian virus tumours, but to the demonstration of a common virus origin for two whole classes (chemically induced and sporadic) of mouse tumours hitherto considered unrelated to the known virus sarcomas of birds, or to the Shope papilloma or the Bittner mammary cancer.

In the second place we have subjected the tumour tissue to various processes besides refrigeration which are known to kill mouse embryo cells, such as fine mincing and dispersion, dilution with glucose, with cystein, buffering to pH6, storage in glycerol and treatment with distilled water. None of these procedures affect the activity of the refrigerated tissue, which remains as great as or greater than that of the fresh tissue. Thirdly, we have made histological examinations of frozen tissue implants at intervals and have examined the material itself, finding, however, neither any evidence for survival of cells, nor conclusive proof of their total destruction, since one cannot be certain that every cell has been examined.

Finally, being well aware that to all these methods of approach might be opposed the objection of the remote possibility of the exceptional survival of a few cells, we have proceeded to dry the refrigerated tissue completely to dust *in vacuo* at a temperature of $-25°$ C. This is universally allowed to be lethal to both normal and tumour cells, yet we have obtained highly active dry material from all the three tumours studied. It therefore appears impossible to avoid the conclusion that from both the chemically induced and the sporadic sarcomas a cell-free agent can be recovered, which on injection into appropriate mice is capable of starting the tumour afresh. From its resistance to extreme cold, to glycerol and to drying the agent seems most likely to be a virus, and since a virus origin is undoubted for many avian sarcomas, this is less surprising than would be a non-virus origin for mammalian sarcoma.

Our experiments will now be described in detail.

I. *The tumours used.*

(1) A sarcoma (C48) of the inbred strain C57 Black. This tumour was obtained by the injection of an oily solution of methylcholanthrene in 1941;

it has been propagated by grafting during the past seven years. During this period attempts had been made again and again to propagate the tumour with cell-free filtrates and with dry tissue, and all had previously failed.

(2) A sarcoma of the strain R3. This arose in the stroma of a spontaneous mammary carcinoma which was transplanted by R. J. Ludford. The sarcoma which formed from the stromal connective tissue was separated from the mammary cancer by Ludford by early transplantations by which the slower growing carcinoma was lost.

(3) A similar sarcoma derived from the stroma of a carcinoma of the strain C3H. This also was obtained as a pure sarcoma by Dr. Ludford.

All three tumours are soft, rapidly growing sarcomas. The R3 sarcoma is the most malignant, the methylcholanthrene tumour the least.

II. *Simple refrigeration experiments.*

The technique was similar for all the experiments. The tumour was dissected and minced, either as finely as possible with scissors or in a Craigie mincer (Craigie, 1949). The mince was drawn up in a syringe with a wide bore needle and put into sterile ampoules, 1 c.c. in each. Occasionally it was diluted with a little saline. The ampoules were then sealed, labelled and frozen to −79° C. by immersion in a solution of solid CO_2 in cellosolve. The temperature of the ampoule was sometimes dropped slowly and sometimes rapidly. No difference in results was noticed. so that no allowance need be made for thermal shock. The ampoules were then transferred to a storage cabinet kept at −79° C. After an appropriate interval of hours, days or months, an ampoule was taken out, thawed to room temperature, opened, and 0·05 c.c. or less of the contents inoculated subcutaneously in the right flank of mice of the appropriate strain. When these ampoules were opened it was always noted that a physical change had taken place in their contents, which had become more homogeneous, softer and slightly sticky after freezing. Twenty-four experiments were made with inoculations at varying intervals with uniformly good results (Table I). It will be noted that the time frozen makes no difference to the results. In the last experiment on the list the material had been frozen 201 days, and was inoculated not only into its mouse of origin, but also into four other strains. Tumours appeared in all four strains.

III. *Refrigeration in various media.*

From experiments with mouse embryo tissue (Mann, 1949) we know that treatment with glucose (without refrigeration) reduces the activity of normal mammalian cells, that storage in glycerol at 4° C. kills them, and that a combination of dextrose and cystein severely damages them, while freezing alone for one hour kills them all. Various experiments with tumour cells using glycerol, dextrose, cystein, and distilled water with and without freezing were therefore performed. Table II shows that these procedures, known to kill normal cells, have no effect on the activity of tumour tissue, dextrose indeed appearing definitely beneficial (Craigie, 1949).

It will be noted that the results in Table I and Table II are very similar, and are consistent with the presence of a tumour agent other than a living mammalian cell. As an example of the amount of manipulation which such tumour mince will stand we can cite the protocol of an experiment with the C48 tumour, carried

TABLE I.—*Frozen Sarcoma Tissue Inoculated Subcutaneously.*

No.	Strain.	Tumour.	Time frozen.	Number inoculated.	Number of tumours.	Approximate time of appearance.	Remarks.
M54	C57	C48	24 hours	4	3	2 weeks	No diluent.
„	„	„	7 days	8	8	2 „	„ „
336	„	„	7 „	6	6	2 „	I.P. inoc. 0·03 c.c.
M54	„	„	14 „	8	8	2 „	..
M56	„	„	21 „	4	4	2 „	..
G4	„	„	22 & 29 days (pooled)	12	8	2–3 „	..
M56	„	„	28 days	4	4	2 „	..
M56	„	„	35 „	4	4	2 „	..
„	„	„	42 „	4	3	2 „	..
G6	„	„	49 „	6	6	2 „	..
G13	„	„	51 „	4	4	2 „	..
G19	„	„	88 „	3	3	2 „	..
„	„	„	88 „	3	3	2 „	Kept at 20° for 1 hour.
G27	„	„	106 „	3	3	2 „	..
G10A	R3	R3β R.T.	107 „	3	3	7 days	..
G10B	„	„	107 „	3	3	2 weeks	Cystein added and kept at 20° C. for 40 min.
G29	C57	C48	109 „	3	3	2–3 „	..
G22	„	„	117 „	3	3	2–3 „	..
M90	„	„	147 „	3	3	9 days	..
416	„	„	192 „	6	6	12 „	Diluted Ringer.
418	„	„	198 „	4	4	12 „	..
„	Simpson	„	198 „	4	3	12 „	Crossed strain.
„	S/A	„	198 „	4	4	12 „	„ „
„	R3	„	198 „	4	3	12 „	„ „
456	MRC/Hunt	37s	201 „	5	5	7 „	No diluent.

Note.—No diluent other than a trace of saline or Ringer was added before freezing. After thawing cystein was added in one case. In two cases the material was left on the bench for approximately 1 hour after thawing before inoculating.

TABLE II.—*Experiments with Sarcoma Tissue using Various Diluents with and without Freezing.*

No.	Strain.	Tumour.	Time frozen.	Diluent.	Number inoculated.	Number of tumours.	Time.	Remarks.
368	C57	C48	Not frozen	0·5–0·75 c.c. tumour mince : 9 c.c. of 55% glycerol in Ringer ½ hr. at 4° C.	6	6	18 days	Finely minced.
„	„	„	„	Ditto 1 hr. at 4° C.	6	6	18 „	„ „
„	„	„	„	„ 2 hrs. at „	6	6	18 „	..
„	„	„	„	„ 4 „ „	6	6	18 „	..
364	„	Primary methylch.	„	Cysteinised. 50% glycerol. 4 days at 4° C.	5	1+	11 „	I.P. inoc. washed with saline before inoc.
367	„	C48	„	Cysteinised. 50% glycerol. 6 days at 4° C.	8	8	19 „	I.P. inoc. Not washed.
M84	„	„	„	Diluted 1 in 12 with distilled water for 40 min. at 4° C.	4	4	12 „	..
„	„	„	„	Diluted 1 in 10 with distilled water for 30 min. at 4° C. and 5·3% glucose added	4	3	3 weeks	..
M65A	„	„	1 hour	5·3% dextrose 1 in 1 dilution	6	6	3–4 „	..
„	„	„	3 hours	„ „	6	5	3 „	..
„	„	„	6 „	„ „	6	6	2–3 „	..
„	„	„	9 „	„ „	6	4	3 „	..
„	„	„	12 „	„ „	6	5	3 „	..
„	„	„	24 „	„ „	6	5	3 „	..
G2	„	„	24 „	Dil. 1½ times with 5·3 dextrose. Top layer of fluid taken and dil. 1 in 5	6	6	8 days	..
				1 in 10	6	5	8 „	
				1 in 100	6	0	..	..
				1 in 1000	6	0	..	..
M60	„	„	2 days	As M65A	6	6	4 weeks	..
G1	„	„	3 „	1 in 10 dil. 10% dextrose Ringer	6	2	4–5 „	Surface layer of fluid taken and diluted 1 in 10.
M60	„	„	4 „	As M65A	6	6	3 „	..
M65	„	„	4 „	„	6	6	3 „	..
436	R3	R3β R.T.	7 „	7 c.c. tissue : 10 c.c. 5·3% dextrose	3	3	7–10 days	..
„	„	„	14 „	Do.	3	3	7–10 „	..
„	„	„	21 „	„	3	3	7–10 „	..
M90	C57	C48	22 „	1 in 1 5·7% dextrose	3	3	9 „	..

TABLE II.—(*cont.*).

No.	Strain.	Tumour.	Time frozen.	Diluent.	Number inoculated.	Number of tumours.	Time.	Remarks.
436	R3	R3β R.T.	28 days	7 c.c. tissue : 10 c.c. 5·3% dextrose	3	3	7–10 days	..
432	„	„	34 „	5 : 2 of 5·3% dextrose	3	3	13 „	..
436	„	„	35 „	7 : 10 : 5·3% dextrose	3	3	7–10 „	..
„	„	„	42 „	„ „	3	3	7–10 „	..
434	C57	C48	53 „	1 in 1 : 5·3% dextrose	3	3	13 „	..
442	R3	R3β R.T.	63 „	„ „	3	3	14 „	..
G14 and 433	C57	C48	74 „	„ „	3	3	14–18 „	Frozen at −79° C. for 28 days. Then at −190° for 1 day. Then returned to −79° for 45 days.
444	R3	R3β R.T.	75 „	5 : 2 of 5·3% dextrose	3	3	7 „	After freezing dil. 1 in 1 with saline. Ulceration occ. early, but healed and tumours grew.
403	C57	C48	83 „	1·5 : 5 in Ringer and then 1 : 0·25 of 5·3% dextrose	8	6	10 „	..
G11A	„	„	113 „	Dextrose 1 in 1 added after thawing	4	4	2–3 weeks	..
440	„	„	142 „	1 : 1·5 of 5·3% dextrose	3	3	2 „	..
413	R3	R3β R.T.	155 „	10% dextrose 1 in 1	6	6	11 days	..

TABLE III.—*Experiments with Dried Refrigerated Tissue.*

No. of Experiment.	Tumours and strain of mouse.	Time frozen before drying.	Details.	Number inoculated.	Number of tumours.	Time of appearance.
406	C48 sarcoma in C57 Black	35 days	Cysteine added after drying	7	6	17 days
G13B	„ „	52 „	1 c.c. 5% dextrose added before drying and cystein after	18	13	16–31 „
434	„ „	53 „	Cystein and Na Bic. added after drying to pH 7·5 and pH 8·5 in some	12	9	16 „
G26	„ „	105 „	Cystein added to pH 7·5 and pounded in mortar	8	1	24 „
436	R3β R.T. in R3 mice	28 „	Cystein added after drying	6	6	11 „
432	„ „	34 „	Frozen in dextrose. Cystein added after drying	10	10	13 „
444	„ „	75 „	Cystein and dextrose added after drying	6	6	11 „

out on October 23, 1948. One g. of minced tumour, which had been stored at −79° C. for three months, was taken and put in a sterile mortar. To this was added 3 c.c. Sörensen phosphate buffer pH 6·0. The mixture of tissue and buffer solution was gently rubbed with a sterile pestle to secure perfect mixing. The semi-fluid mixture was again frozen hard by putting the mortar in a mass of CO_2 snow and left for 30 minutes. The mortar was then taken out of the CO_2 snow and solid frozen tissue and buffer pounded vigorously with the pestle, to break up as far as possible any remaining whole tumour cells. Thirty c.c. of cold distilled water was added, and when the mixture was finally liquid it was spun for 15 minutes at 10,000 revolutions a minute. The supernatant fluid was reddish in colour and was of a pH 6·0; the deposit, firmly fixed as a thick film on the bottom of the centrifuge tube, was perfectly bleached and white. After washing the deposit with saline, it was emulsified in 3 c.c. of saline (·85 per cent NaCl in distilled water) and the emulsion injected into six C57 mice. All the mice developed typical C48 tumours, which were large on the 14th day. Films of the emulsion were examined under the microscope and in none could a whole cell be found; all that could be seen consisted of naked nuclei and separated masses of cytoplasm. Experiments such as this do not appear to lend any support to the belief that the tumours started by frozen tissues are transplants, i.e. due to division of surviving tumour cells.

IV. *Histological investigations.*

Our histological investigations, though necessarily inconclusive as are all opinions based on sectioning or on examinations of random samples of a cell suspension, have also failed to support the idea of cell survival. Samples of tissue mince show before refrigeration that many of the cells are damaged, even by the mincer. After simple freezing there are even fewer apparently intact cells, while after treatment with glucose or with distilled water profound changes appear affecting both nucleus and cytoplasm. A detailed study of these changes is being made by one of us and will be published later.

A series of sections of implants of tumour tissue (both sarcoma and carcinoma) frozen to −79° C. for one hour was made at intervals of 2, 3, 4, 5, 6 and 7 days. Microscopically the mass appeared completely necrotic on the second day. Sections of such frozen sarcoma tissue show on the second day merely a mass of necrotic tissue surrounded by the normal subcutaneous tissue of the recipient mouse. By the fourth day much of this tissue is being absorbed and removed by the host, and on the fifth and sixth days and subsequently, the beginnings of a new sarcoma can be seen in the connective tissues surrounding the necrotic mass. The appearances do not suggest survival of any of the implanted tissue, but rather a fresh sarcomatous reaction of the encapsulating subcutaneous tissues of the host. In the case of frozen carcinoma also no living tumour cells were seen, and by the seventh day the necrotic mass was encapsulated and shrunken. There was no sign of a new tumour.

Dried tissue examined microscopically showed only a mass of débris and shrunken nuclei.

V. *Experiments with dried tissue.*

Our results thus far appeared consistent with the view that freezing to −79° C. for long periods kills the cells and liberates a virus which is active, but probably

only stable at low temperatures (since refrigerated tissue left on the bench gradually lost its activity during 12 to 24 hours and frozen tissue incubated at +37° C. became inactive in two hours). It would therefore appear likely that if highly active refrigerated tissue could be dried without raising the temperature infective dried material could be obtained. This has been successfully carried out as shown in Table III.

The R3 tumour appeared to be so extremely active in one batch (Experiment 444) that the violence of the response produced ulceration. There was no evidence of infection and the tumour grew rapidly from the edges of the ulcers, infiltrating the muscle. In every way this tumour seemed more active than the C48, so attempts were made to dry it without preliminary freezing. They were successful, as was also a similar experiment with the C3H tumour which was dried fresh by Dr. Craigie. It has not so far been possible to obtain active dried material from the C48 without preliminary storage at −79° C.

A brief account of the method of drying must be given, though for details of the apparatus used Craigie's paper (1949) should be consulted. The tissue to be dried is either taken from the storage ampoule or, if freshly minced, diluted with an equal volume of 5·3 per cent dextrose and is run into the drying flask. It should be distributed as evenly as possible over the bottom of the flask in a thin layer. The flask is immediately cooled down to −25° C. by plunging it into cellosolve at this temperature. It is then connected to the apparatus and the pump started. The time is noted and the pump run until the indicator falls

TABLE IV.—*Experiments with Fresh Dried Tissue.*

Experiment No.	Tumour.	Condition of tissue.	Details.	Number of mice inoculated.	Number of tumours.	Time of appearance of tumours (days).
437	R3β R.T.	Fresh mince	Diluted 4 gm. to 10 c.c. of 5% dextrose. 3 c.c. cystein added after drying	10	8	14–33
436	R3β R.T.	Do.	As above	6	1	14
C80	C3H R.T.	„	Diluted 1 in 10 with 5% dextrose, 5% blood; 1·5 c.c. of M/150 cystein pH 7·5 added after drying	12	6	15–27

to zero. Pumping is then continued for a further half or one hour to be absolutely certain of the removal of the last traces of water. The time taken to reach zero averaged 113 minutes in eight experiments. When drying is complete the vacuum is slowly released. The flask is removed and 2–3 c.c. of a 1 in 500 neutral solution of cystein is run slowly in. This rapidly produces an even emulsion, 0·2–0·5 c.c. of which is injected subcutaneously in each mouse.

Before this method of drying was evolved, many unsuccessful experiments with these tumours, using the Knox method of drying, had been carried out. Even now a successful result is not obtained in every case, and much further experimentation will be required to determine the optimum conditions. We do

not know whether the length of time of preliminary freezing has any effect, but it is likely that it has, since our best results were obtained during the second month of freezing. It may also be that each tumour has its optimum freezing time and that many may be dried from the fresh state, the only freezing required being that during the drying. It may be that each tumour passes through cycles of activity, so that it can sometimes be successfully dried and sometimes not; this occurs even with the Rous tumour. It may be that other diluents may be found to give better results than dextrose and cystein. It is now, however, undoubted that mammalian sarcomas, both chemically induced and spontaneous, may be propagated by dried tissue. The resultant tumours reproduce with extreme fidelity the histological and biological characters of the originals.

An experiment still in progress may be mentioned. An R3β R.T. tumour was successfully dried after 34 days' freezing. The resultant tumours were dried from the fresh state. Tumours were again obtained, and are now being frozen for a further drying. Thus the virus can, as it were, be passaged through a succession of freezings and dryings which completely exclude persistence of any of the original cells. These experiments therefore justify the conclusion that cancer, as a pathological process, is the effect of the parasitization of a cell with a specific virus.

SUMMARY AND CONCLUSIONS.

1. Sarcomas of inbred mice have been investigated for their behaviour on freezing to −79° C.

2. Minced sarcoma tissue retains its power to start fresh tumours after storage at −79° C. for an indefinite period.

3. Such refrigerated tissue can be successfully dried and tumours started afresh with the dried material.

4. Some sarcomas of mice can be propagated by dried tissue without preliminary freezing.

5. Both chemically induced and sporadic sarcomas can be transmitted by frozen and dried material.

6. The continuing cause of these tumours is probably a virus which is stable at −79° C.

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THE PRESERVATION OF SUSPENSIONS OF TUMOUR CELLS IN DEXTROSE AT LOW TEMPERATURES.

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ALTHOUGH convenient and entirely suitable for the purposes for which they are employed, the usual methods of maintaining and utilizing transplantable tumours are not well adapted to special needs in experimental oncology, particularly when quantitative refinements and the desirability of repeating experiments with aliquot portions of the same tumour suspension are important considerations. The basis of any method which might meet such requirements must necessarily be one in which a number of separate and similar portions of a tumour can be preserved with some assurance that different portions tested at different times will exhibit similar activities. This means that the tumour tissue must be reduced to a fine state of division and homogeneous suspension and subjected to a method of preservation whereby its capacity to induce tumours remains constant for a long time.

It is well known that growth may be obtained on transplantation of frozen and thawed tumour tissue. Although Breedis and Furth (1938) demonstrated the feasibility of preserving neoplastic cells in the frozen state for periods up to 400 days, this useful method does not seem to have been adopted to the extent that might have been expected. Much of the experimental work reported on the production of tumours with frozen and thawed material is concerned with the interpretation of the survival of activity and it is clear that under certain conditions malignant cells and normal skin (Briggs and Jund, 1944) will survive freezing temperatures for some time. It seems probable that lack of suitable refrigeration facilities, together with a lack of confidence in a phenomenon by means fully understood, have prevented any extensive practical use of tumours preserved in the frozen state. Breedis (1942) showed that mouse leukemic cells are inactivated if frozen too quickly in the temperature range between 0° C–15° C., but remarks that different types of cells may require different conditions for survival. Such a variable dependence of survival on the rate of freezing and perhaps that of thawing does not exactly recommend preservation by freezing as a reliable practical method.

Most of the observations on the survival of tumours at low temperatures relate to pieces of tumour frozen *en masse*. There are few reports on cell suspensions, apart from the work of Breedis (1942) on leukemic cells. Mider and Morton (1939) found that saline suspensions of tumour cells were more readily inactivated than portions of tumour. It might be expected that cells exposed to an artificial fluid environment are more likely to be killed by freezing than are cells situated in the interior of a piece of intact tissue, because the former are

more exposed to the mechanical effects of ice formation and to the strong concentrations of inorganic salts induced between the initial freezing point and the freezing temperature of their eutectic solutions. Nevertheless, as has been indicated, various technical and quantitative considerations suggested the desirability of investigating the survival of finely divided tumour tissue suspended in artificial fluids and kept frozen at low temperatures. Some of the observations which have been made in the course of this study are presented in this paper which is necessarily of the nature of a preliminary communication.

MATERIALS AND METHODS.

Tumours.—The tumours studied are listed in Table I. Most of the observations have been made using the C3H sarcoma referred to by Gye, Begg, Mann and Craigie (1949).

Preparation of tumour suspensions.—The tumours were reduced to the necessary state of subdivision by means of the pressure mincer described by Craigie (1949) and the mince dispersed immediately in diluting fluid. The diluting fluids were freshly prepared from reboiled sterile distilled water and concentrated preparations of the required solutes. In experiments in which different diluting fluids were compared, a low primary dilution was first made in fluid of suitable composition and from this the secondary dilutions in the other fluids were prepared. The suspensions were distributed in suitable amounts to 3 or 5 ml. glass ampoules, which were immediately sealed.

Freezing.—The sealed ampoules, fitted with lead weights, were immersed in an ethyl cellosolve bath in a thermos jar. When slow freezing was employed the bath was cooled by the addition of CO_2 ice. On other occasions it was precooled to the required temperature. After freezing had taken place the bath was cooled to — 79° C. before the ampoules were removed and transferred to CO_2 ice.

Storage.—The ampoules were stored in a heavily insulated box kept charged with CO_2 ice. This box is so constructed and vapour sealed that the only exit for the cold CO_2 vapour is the narrow space around the edges of the horizontal insulated lid.

Thawing.—When required for test an ampoule was transferred directly from CO_2 ice to water at 37° C. and kept in gentle motion until all but a small portion of the contents had thawed. While this was being done the ampoule was watched carefully for gas bubbles indicating faulty sealing or the presence of a crack in the glass.

It is to be noted that this thawing schedule is empirical and the influence of rate of thawing remains to be investigated.

Inoculation.—Injections of 0·1 c.c. were given subcutaneously in the appropriate strain of mice. Except in limited experiments, four sites in the anterior abdominal wall were used, these being approximately the centres of the upper and lower right and left quadrants.

The survival of tumour suspensions in salt solutions.

When a solution of sodium chloride is frozen at a temperature above — 21°C. ice crystallizes out and the salt is concentrated as a strong solution (30 per cent) between the crystals. If a cell suspension in 34 volumes of 0·85 per cent NaCl is frozen at — 20° C. the volume of concentrated salt solution formed will be

approximately equal to that of the cells. Preliminary experiments were, therefore, undertaken to determine the inactivating effect of strong NaCl and Ringer salt concentrations on fresh, unfrozen sarcoma cells. In some of these the cells were held at 0° C. in the strong salt solutions, in others the material was held in the frozen state for several hours at selected temperatures above and below that of the eutectic of NaCl; the results showed that although complete inactivation might require several hours, the initial rate of inactivation referable to electrolyte concentration was undesirably high.

Survival of tumour suspensions in dextrose solutions.

In view of the difficulties that might be expected to arise in attempts to obtain adequate preservation of activity in tumour suspensions frozen in inorganic salt solutions, the possibility of substituting a suitable non-electrolyte was considered. Accordingly it was planned to test a variety of water-soluble organic materials, but dextrose, the first of these selected for test, was found to have such a favourable effect that no attempt has yet been made to find substances more suitable for the purpose.

Because of the varied nature of the comparative tests which have been carried out and the introduction of a number of improvements in technique, it is not possible to present a synopsis of the results on a quantitative basis. Table I provides a brief summary which, at best, merely shows that there is no contra-indication to the use of dextrose solution as a suspending fluid. The dilutions of the tumour suspensions, as prepared for freezing, ranged from 1 in 2·5 to 1 in 100.

TABLE I.

Tumour.	Suspending fluid.	Number of lots frozen.	Number active after thawing.	Number inactive after thawing.
C3H sarcoma	Saline	6	5	1
	Hypotonic NaCl	2	1	1
	Ringer	7	7	0
	Hypotonic with cysteine phosphate buffer	8	6	2
	20% gelatin	2	1	1
	20% peptone	1	0	1
	Dextrose solutions	53	53	0
	Glycerine and dextrose	3	3	0
R3 sarcoma	Dextrose solutions	7	7	0
2146 sarcoma	Ringer	1	1	0
37S sarcoma	Dextrose solution	1	1	0
C3H mammary carcinoma	,, ,,	9	9	0
Totals		100	94	6

Of 70 lots of sarcoma and carcinoma suspension kept frozen in dextrose for periods of a few days to many months, all proved active on thawing. In all comparative tests the dextrose preparation was more active than the correspond-

ing preparation from which it was omitted. The protective effect of dextrose was evident at concentrations from 3 per cent to 40 per cent. The optimum, for the C3H sarcoma at least, appears to be between 5 and 10 per cent and 5 per cent dextrose, which is approximately isotonic, has been adopted for routine purposes.

Table II shows the results obtained in an experiment in which hypotonic NaCl, isotonic NaCl, Ringer and 5 per cent dextrose solutions were compared as suspending fluids for the C3H sarcoma.

TABLE II.—*C3H Sarcoma Suspension.*

Suspending fluid.	Treatment of suspension in dilution 1 in 30.	Time frozen.	Diluent.	Response to 0.1 c.c.			
				Dilution.	Mouse group.	Number of takes.	Mean lag.
0·056% NaCl	0° C. for 1 hr.	0	R	1 : 300	I	5/10	12·2
			CR	„	II	8/10	14·25
0·85% NaCl	„	0	R	„	I	10/10	11·66
			CR	„	II	7/10	13·33
Ringer	„	0	R	„	I	10/10	10·6
			CR	„	II	10/10	10·5
5% dextrose with 0·056% NaCl	„	0	R	„	I	10/10	9·8
			CR	„	II	9/10	11·25
0·056% NaCl	Frozen after 0° C. for 1 hr.	11 days	S	1 : 150	III	0/10	—
			CS	„	IV	0/10	—
0·85 NaCl	„	„	S	„	III	0/10	—
			CS	„	IV	0/10	—
Ringer	„	„	S	„	III	2/10	17·5
			CS	„	IV	6/10	17·4
5% dextrose with 0·056% NaCl	„	„	S	„	III	7/10	14·7
			CS	„	IV	10/10	13·6
5% dextrose with 0·056% NaCl	„	21 days	S	1 : 300	V*	8/10	17·6
			CS	„	V	8/10	16·75
			R	„	V	8/10	17·8
			CR	„	V	8/10	16·25

S = 0·85 per cent NaCl ; R = Ringer ; C = M/150 cysteine HCl, M/150 NaOH, M/150 Na_2HPO_4, pH 7·5.

Four comparative injections were made in each mouse and the figure given in the column headed " mouse group " indicates the arrangement of injections, e.g. all four suspensions were compared in all 10 mice of Group I.

* One mouse negative to all injections.

Mean lag ; see text.

The tumour mince was first thoroughly mixed with an equal volume of 0·85 per cent NaCl and the mixture was diluted 1 in 15 in the stated diluents. The dilution of the sarcoma tissue was thus 1 in 30. After distribution to ampoules the suspensions were held at 0° C. for 1 hour, when one ampoule of each lot was opened for dilution and injection, while the remainder were frozen. Ampoules of the frozen suspensions were thawed and tested 11 days later. It will be noted that the saline suspensions were inactive at the dilution tested, while the Ringer suspension was less active than the dextrose suspension. A further test of the dextrose suspension was made after it had been kept frozen for 21 days. As far as can be estimated from the available data on the relationship between log. dose of frozen material and lag period, the activity of the frozen and thawed dextrose preparation was approximately 17 per cent of that of the control dextrose preparation. The term lag period, as used here, means the time interval in days elapsing between injection and the first appearance of growth at the sight of injection.

The survival of tumour suspensions in other suspending fluids.

The various suspending fluids tested are indicated in Table I. Some of the observations are incomplete and do not merit comment. The inactivation observed with gelatine and peptone cannot be explained, but is not attributable to faulty sealing of the ampoules. The corresponding dextrose controls were of usual activity.

The survival of activity of C3H sarcoma suspension frozen in mixtures of dextrose and glycerol is worthy of note. Details of one experiment are given in Table III.

TABLE III.—*C3H Sarcoma Suspension.*

Frozen in dilution of 1 in 12.5 in—	0.1 c.c. injected subcutaneously after thawing and further dilution.					
	Dilution tested.	1 : 12.5.	1 : 125.	1 : 25.	1 : 17.5	1 : 70
(*a*) 10% dextrose	Number of takes	6/6	5/6	—	12/12	12/12
	Mean lag in days	8·0	9·8	—	4·17	8·73
(*b*) 10% dextrose with 20% glycerol	Number of takes	6/6	6/6	12/12	—	—
	Mean lag in days	8·5	9·2	4·0	—	—
(*c*) 10% dextrose with 40% glycerol	Number of takes	6/6	5/6	—	12/12	12/12
	Mean lag in days	11·0	13·6	—	4·08	7·08
Time frozen with CO_2 ice		4 days		222 days	253 days	
Diluent		Ringer		Cysteine-phosphate saline.	Cysteine-phosphate saline.	

— = not tested.

C3H sarcoma mince was suspended to a dilution of 1 in 12·5 in the following fluids: (*a*) 10 per cent dextrose, (*b*) 10 per cent dextrose with 20 per cent glycerol, (*c*) 10 per cent dextrose with 40 per cent glycerol. The first test was made after the suspensions had been kept frozen for 4 days. The lag periods observed in this test are not comparable with those in the later tests because of changes in technique of thawing, diluting and injecting, and also because of the use of a cysteine-phosphate saline mixture in the later tests. However, it would appear legitimate to compare the activity of the material frozen for 253 days in the mixture of dextrose and 40 per cent glycerol with the average activity of fresh C3H sarcoma suspension in Ringer. A lag period of 7·1 days corresponds with a dilution range of 1 in 170 to 1 in 300 of fresh C3H sarcoma suspension. Since the glycerinated suspension gave a mean lag period of only 7·08 days in a 1 in 70 dilution it might be concluded that 25 per cent of the tumour cells viable in

EXPLANATION OF PLATE.

FIG. 1.—Cells of C3H sarcoma suspended in Ringer. Phase contrast. × 460. Note nuclear detail.

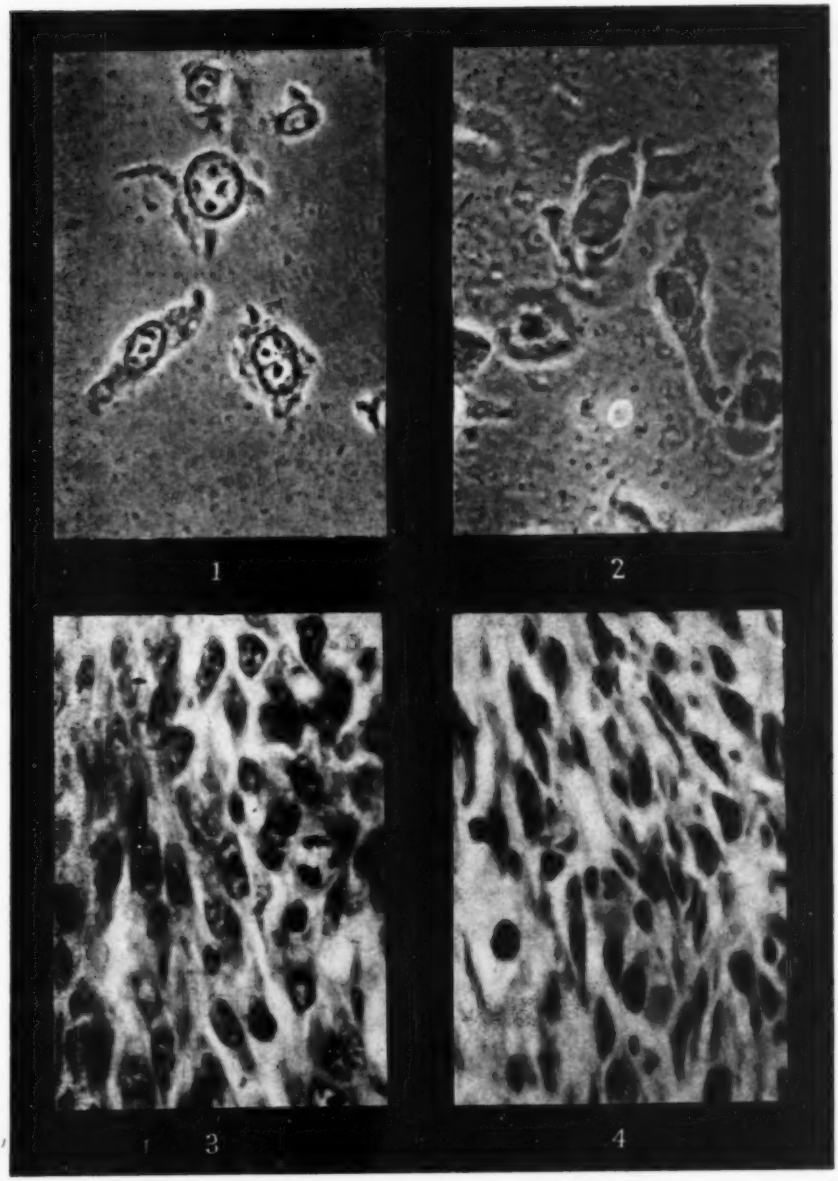
FIG. 2.—Same preparation of cells as shown in Fig. 1 after dilution with 4 volumes of 5 per cent. dextrose. Phase contrast. × 460. Note absence of nuclear detail. Three cytoplasmic granules appear above the nucleus of the cell at lower right.

Note.—All photographic manipulations were carried out in such a way that the relative densities in Fig. 1 and 2 are strictly comparable.

FIG. 3.—C3H sarcoma fixed in Zenker, stained with haematoxylin and eosin. × 690. For comparison with Fig. 1.

FIG. 4.—C3H sarcoma, from same tumour as Fig. 3, soaked in 5 per cent dextrose for one hour before transfer to Zenker, stained with haematoxylin and eosin. Representative field from peripheral zone showing the effect of exposure to dextrose. × 690. For comparison with Fig. 2.

Note.—The increased magnification of Fig. 3 and 4 does not compensate for the shrinkage due to fixation and dehydration of the tissue.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the tumour mince survived the subsequent treatment to which they were subjected, but for the prevailing opinion that fluids containing glycerol do not favour the retention of cellular functional activity.

Survival of other tumour suspensions in dextrose solutions.

Gye, Mann and Begg (personal communication) have found that the following additional tumours may be satisfactorily preserved as frozen dextrose suspensions : MC 48 (C 57 strain), 63 carcinoma, R III sporadic carcinoma, Jensen's rat sarcoma, Walker rat tumour, Dael's sarcoma of the guinea-pig.

COMMENT.

Although it has been empirically established that the use of dextrose solution as a suspending fluid provides a reliable method of preserving mouse sarcoma and carcinoma suspensions for considerable periods in the frozen state, much detailed quantitative work would seem to be indicated. Further investigation is desirable not only with a view to improvements that might facilitate studies in which the use of preserved tumour material is preferable, but also with the object of determining the mechanisms of survival of activity. The variety of physical and physico-chemical factors which may be involved and the potential complexity of their interplay need not be detailed, but some of the points which require investigation from the practical point of view may be mentioned.

They are as follows :

1. A comparison of other sugars with dextrose.
2. The influence of rate of cooling on survival. One of the advantages of dextrose appears to be that it renders this factor less important, but the impression requires confirmation.
3. The influence of temperature of preservation. CO_2 ice and special low temperature machines provide relatively costly methods of refrigeration. The value of dextrose, in part at least, may be referable to its influence on electrolyte or ion distribution. If so, the development of more efficient suspending fluids may make it possible to obtain adequate preservation with commercially available equipment operating at a minimum of — 18° to — 20° C.
4. The influence of hydrogen ion and oxidation reduction potentials.
5. The influence of rate of thawing.
6. The development of a suitable fluid for dilution of thawed material. Tumour suspensions which have been frozen may show a definite drop in activity in 15 to 30 minutes after thawing. The rate of inactivation depends, in part, on the degree to which the material is diluted. It will have been noted from Tables II and III that cysteine phosphate buffer mixtures have been used as diluting fluids. The experimental evidence for the use of diluting fluid of this composition is contradictory and suggests a different approach to this problem.

Another matter which merits further investigation is the effect of dextrose on the nuclei of tumour cells as viewed with the phase contrast microscope. This is illustrated in Fig. 1 and 2. When sarcoma cells are transferred to dextrose solution nuclear detail becomes very rapidly obscured, nucleoli and chromatin

masses disappear and the nucleus assumes a homogeneous hyaline or vitreous appearance. This might be regarded as an obscure optical effect under phase contrast, but for the fact that a pyknotic change is evident in sarcoma tissue soaked in dextrose solution prior to fixation (Fig. 3 and 4). The nuclear alteration produced by dextrose persists after freezing and thawing, and is reversible by the addition of NaCl solutions. Sufficient cover slip pressure on a sarcoma cell viewed by phase contrast will result in the disappearance of nuclear detail, but this in no way accounts for the changes observed with dextrose, which produces its effects both on nuclei in fresh intact cells and nuclei stripped of cytoplasm.

Glycerol and distilled water have an effect on the appearance of the nucleus similar to that of dextrose, although their other effects on the cell, osmotic swelling and dehydration respectively, are entirely different. In a balanced salt and dextrose solution the appearance of the nucleus may be altered by adjustment of the hydrogen ion concentration, nuclear detail fading on the alkaline side.

SUMMARY.

1. After preservation in the frozen state, sarcoma and carcinoma suspensions in dextrose solution show a greater and more consistent retention of activity than suspensions prepared with saline and Ringer. The degree of survival of activity in dextrose preparations at a low temperature is adequate for experimental work requiring the use of transplantable tumours and for some purposes may offer a more convenient source of material than fresh tumour tissue.

2. A C3H sarcoma suspension frozen in dextrose and 40 per cent glycerol was found to be highly active after storage for 253 days.

3. Reference is made to changes induced by dextrose in the cell nucleus as seen under phase contrast illumination.

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THE EFFECT OF A RESTRICTED DIET ON MITOTIC ACTIVITY IN THE MOUSE.

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In a recent publication (Bullough, 1949) it has been shown that epidermal mitotic activity in the adult male mouse is strongly influenced by the concentration of sugar or glycogen in the tissues. The mitosis rate rises to a high level when the blood sugar is deposited in the tissues during sleep, and similarly it can be raised artificially by injections of carbohydrate. Conversely, a reduction in the blood sugar level, induced by insulin injections, causes an almost complete cessation of epidermal mitotic activity, and the same effect is caused by phloridzin, which interferes with the phosphorylation of sugar.

Since insulin and phloridzin both exert their mitosis depressing effect by reducing the availability of sugar, it appeared probable that a similar depression would also be induced by starvation, and in view of current interest in the effects of nutrition on the development of spontaneous and induced tumours (Tannenbaum, 1947), it was considered important that this aspect of the problem should be investigated.

MATERIAL AND METHODS.

The mice used in this investigation were Strong CBA males of between three and six months of age. Since weaning they had all been allowed to feed *ad libitum*, and thus they were all heavy mice in good condition. Until the experiments began they received a mixed diet of maize, dog biscuit, rat cake soaked in cod-liver oil, and chopped carrots, and this food was given to them daily between 09.00 and 10.00 hours. The laboratory was artificially lighted between 09.00 and 18.00 hours. These details of times are important, since the disturbances associated with them determine the timing and form of the diurnal mitosis cycle (Bullough, 1948*a*, *b*).

The mitosis rate was measured in the ear epidermis by the earclip technique. Small pieces of ear, each about 3 mm. square, were cut away at intervals by means of a conchotome. These clips were then fixed in Bouin's alcoholic fluid, and cut into sections 7μ thick. All stages of mitosis were counted in unit section lengths of 1 cm., and from each earclip 10 such counts were made and the average taken. As each experimental group consisted of earclips from ten mice, ten average figures were obtained from which the mean and standard error were calculated according to the method recommended for small samples by Simpson and Roe (1939).

For comparison, blood sugar estimations were also made. Two samples,

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each of 0·1 c.c., were taken from each mouse after it had been pithed and had had its throat cut open. The sugar concentration was estimated by a modification of the Hagedorn and Jensen technique, and it is a great pleasure to record here the assistance received from Dr. A. Jordan, who arranged for this to be done in the laboratories of the Sheffield Royal Infirmary. From the two results obtained from each animal an average was taken, and from the ten averages from each group of mice the mean and standard error were calculated.

RESULTS.

1. *The effects of starvation.*

In these first experiments three groups, each of ten male mice, were deprived of food, but not water, while a fourth group, also of ten male mice, was used as an untreated control. The experiments commenced at 10.00 hours, by which time one experimental group had been without food for 12 hours, the second for 24 hours, and the third for 36 hours. Earclips were taken hourly from 10.00 to 16.00 hours, so as to include the early afternoon sleep period when mitotic activity is normally high. It should be noted that even 36 hours of starvation did not affect the observance by the mice of this period of rest. The mitosis counts obtained from the control and experimental groups are given in Table I and in Fig. 1.

TABLE I.—*The Effects of Various Periods of Starvation on the Epidermal Mitosis Rate of Adult Male Mice.*

Time of day.	Average numbers of mitoses per cm. length of sections cut 7μ thick.			
	No starvation.	12 hours' starvation.	24 hours' starvation.	36 hours' starvation.
10.00	2·9 ± 0·24	1·5 ± 0·29	1·0 ± 0·13	0·1 ± 0·06
11.00	2·3 ± 0·19	0·4 ± 0·09	0·9 ± 0·15	0·2 ± 0·07
12.00	3·1 ± 0·23	0·5 ± 0·11	0·5 ± 0·11	0
13.00	4·5 ± 0·17	1·6 ± 0·26	0·7 ± 0·12	0·1 ± 0·05
14.00	8·3 ± 0·30	4·1 ± 0·35	2·0 ± 0·21	0·2 ± 0·07
15.00	6·6 ± 0·24	1·9 ± 0·28	1·1 ± 0·19	0·4 ± 0·09
16.00	4·3 ± 0·19	1·3 ± 0·18	1·1 ± 0·17	0·1 ± 0·06
Totals	32·0	11·3	7·3	1·1

For comparison, other mice were killed in groups of ten at 10.00 hours after the same treatments, and estimations were made of their blood sugar levels. These are recorded in Table II.

TABLE II.—*The Effects of Various Periods of Starvation on the Blood Sugar Level of Adult Male Mice Killed at* 10.00 *hours.*

Average blood sugar levels in mg. per 100 c.c.			
No starvation.	12 hours' starvation.	24 hours' starvation.	36 hours' starvation.
164·3 ± 3·11	155·3 ± 3·29	127·7 ± 3·57	103·0 ± 2·96

The figures for the various groups of untreated control mice show that these animals had a normal mitosis rate and a normal blood sugar level (Bullough, 1949). The hour-to-hour variations in the numbers of observed mitoses are part of the diurnal cycle, and, at least in the mice of this colony, it is normal for maximum mitotic activity to develop at 14.00 hours, when the animals are asleep.

After 12 hours of starvation the blood sugar level remained fairly high, but the drop in the mitosis rate was pronounced. However, during the afternoon sleep period there was a marked rise in mitotic activity at 14.00 hours to a figure of 4·1 ± 0·35, which is about half the figure reached by the well-fed controls. After 24 hours' starvation the blood sugar level had fallen seriously, and mitotic activity had reached a very low level. Again, however, there was a slight response to the period of rest, so that at 14.00 hours a figure of 2.0 ± 0·21 was obtained. This is about a quarter of the figure for the control animals After 36 hours the blood sugar concentration had dropped to 100 mg. per 100 c.c., and mitotic activity was almost eliminated.

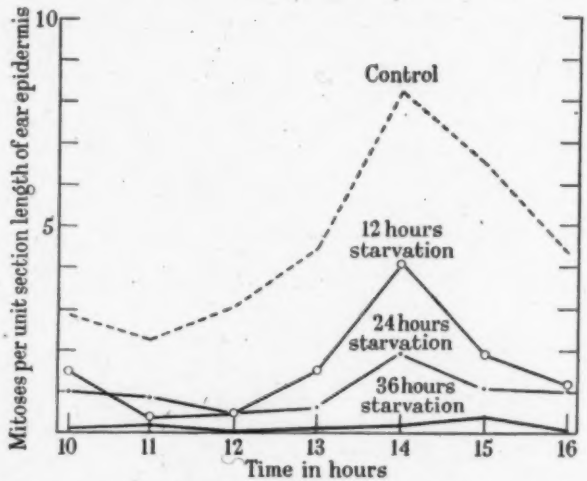

FIG. 1.—The effect of complete starvation on the epidermal mitosis rate of adult male mice.

2. *The effects of a restricted diet.*

Following the experiments of Tannenbaum (1947), who determined the effects of a restricted diet on the development of tumours, observations were next made on the effects of such diets on epidermal mitotic activity. In preliminary experiments determinations were made of the daily food intake of three- or four-month-old Strong CBA males which were allowed to feed *ad libitum* on a diet of rat cake. Two groups, each of five mice, gave the results shown in Table III.

TABLE III.—*The Weights of Rat Cake Eaten Daily by Two Groups, each of Five Males, which were Fed* ad libitum.

Group.	Number of observations.	Daily average in g.	Daily average in g. per mouse.
1	39	18·3 ± 0·34	3·7
2	20	18·1 ± 0·22	3·6

The two results were in strikingly close agreement, each animal eating about 3·6 g. of rat cake per day.

An experiment was then set up with one group receiving 3·6 g. of rat cake per head per day; with a second group restricted to a 66 per cent diet of 2·4 g. per head per day; and with a third group restricted to a 50 per cent. diet of 1·8 g. per head per day. Each group contained ten Strong CBA males aged 4 months, and the animals were fed daily as usual at 09·30 hours. The experiment was also duplicated with all the conditions identical except that the mice

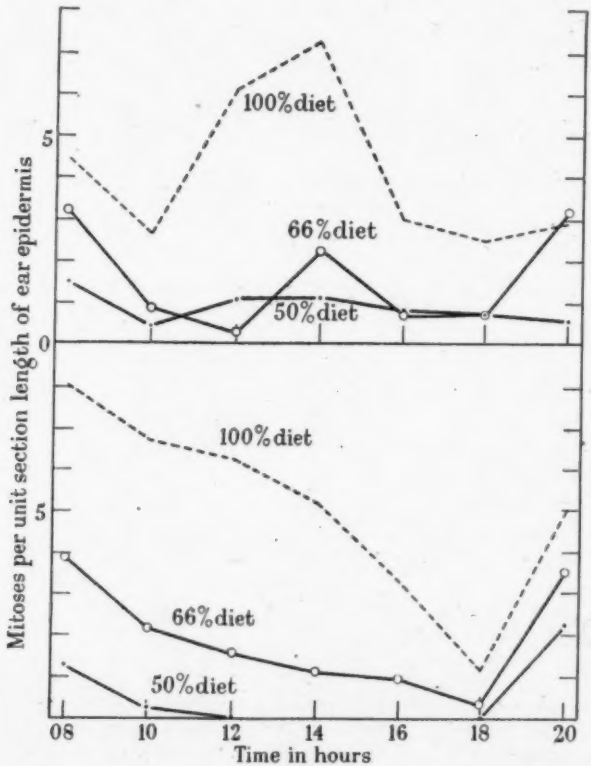

FIG. 2.—The effect of restricted diets on the epidermal mitosis rate of adult male mice fed at 09·30 hours.

were fed daily at 17.30 hours. This was done because it was observed that the mice on the restricted diets ate all their daily rations within an hour of receiving them. Consequently it was thought possible that the blood sugar level and the mitosis rate might rise considerably for a short period after a meal, and, if only morning fed mice were examined, an experiment carried out in the daytime might give misleading or inadequate results.

The various groups of mice were maintained in these conditions for four weeks before the earclips were taken, and during this time the changes in body weight were determined (Table IV).

TABLE IV.—*The Effects of Restricted Diets on the Body Weight of Adult Male Mice.*

Time in weeks.	Average weights per mouse (in g.). Fed at 09.30 hours. 100% diet.	66% diet.	50% diet.	Fed at 17.30 hours. 100% diet.	66% diet.	50% diet.
0	26·2 ± 0·66	29·0 ± 1·01	28·3 ± 1·41	29·6 ± 0·89	29·2 ± 1·59	28·1 ± 1·12
1	28·4 ± 0·76	28·5 ± 0·95	27·4 ± 1·06	29·4 ± 1·07	26·3 ± 0·91	27·7 ± 0·68
2	28·3 ± 1·10	27·3 ± 1·20	25·2 ± 0·87	29·7 ± 0·96	24·5 ± 0·94	21·2 ± 0·49
3	28·0 ± 0·78	26·8 ± 1·03	21·1 ± 0·58	29·1 ± 0·90	21·6 ± 0·47	19·5 ± 0·65
4	29·6 ± 0·83	23·8 ± 0·86	20·0 ± 0·42	30·7 ± 0·81	22·1 ± 0·89	18·9 ± 0·75

At the end of the 4th week the mitosis rates were determined by the removal of earclips at 2-hour intervals from 08·00 to 20.00 hours, and the results obtained are shown in Tables V and VI and in Fig. 2.

TABLE V.—*The Effects of Restricted Diets on the Epidermal Mitosis Rate of Adult Male Mice Fed at* 09.30 *hours.*

Time of day.	Average numbers of mitoses per cm. length of sections cut 7μ thick. 100% diet.	66% diet.	50% diet.
08·00	4·4 ± 0·17	3·2 ± 0·22	1·5 ± 0·07
10·00	2·7 ± 0·14	0·9 ± 0·13	0·4 ± 0·04
12.00	6·1 ± 0·25	0·3 ± 0·08	1·1 ± 0·11
14.00	7·6 ± 0·24	2·4 ± 0·12	1·2 ± 0·13
16.00	2·9 ± 0·18	0·6 ± 0·17	0·7 ± 0·11
18.00	2·5 ± 0·21	0·6 ± 0·09	0·6 ± 0·07
20.00	2·8 ± 0·29	3·2 ± 0·21	0·5 ± 0·05
Totals	28·6	11·2	6·0

TABLE VI.—*The Effects of Restricted Diets on the Epidermal Mitosis Rate of Adult Male Mice Fed at* 17.30 *hours.*

Time of day.	Average numbers of mitoses per cm. length of sections cut 7μ thick. 100% diet.	66% diet.	50% diet.
08.00	8·0 ± 0·19	3·9 ± 0·21	1·3 ± 0·15
10.00	6·7 ± 0·18	2·2 ± 0·17	0·3 ± 0·16
12.00	6·4 ± 0·38	1·6 ± 0·15	0
14.00	5·3 ± 0·21	1·2 ± 0·26	0
16·00	3·3 ± 0·24	1·1 ± 0·11	0
18.00	1·2 ± 0·12	0·4 ± 0·07	0
20.00	5·0 ± 0·27	3·5 ± 0·24	2·3 ± 0·20
Totals	35·9	13·9	3·9

It is evident that a reduced diet causes a pronounced depression of the epidermal mitosis rate, the 66 per cent diet having an effect similar to that of about 24 hours of complete starvation, and the 50 per cent diet to that of about 36 hours of complete starvation. Mice kept on a 66 per cent diet maintained a mitosis rate which, on the average, was less than 40 per cent of that of the well-fed controls, while those fed on a 50 per cent diet had a mitosis rate which was only about 15 per cent of that of the controls. In these respects there was little, if any, difference between the mice fed at 09.30 hours and those fed at 17.30 hours. There were, however, marked differences between the forms of the diurnal cycles which need not be commented on here.

As regards the animals themselves, those fed *ad libitum* and those fed on a

66 per cent diet remained active and in excellent health, but those which received a 50 per cent diet showed signs of ill-health, and it is doubtful whether they could have survived the treatment for more than another few weeks.

At the end of the experiment at 20.00 hours all the mice were killed and blood sugar estimations were made. The results are given in Table VII.

TABLE VII.—*The Effects of Restricted Diets on the Blood Sugar Level of Adult Male Mice Killed at* 20.00 *hours.*

Average blood sugar levels in mg. per 100 c.c.					
Mice fed at 09.30 hours.			Mice fed at 17.30 hours.		
100% diet.	66% diet.	50% diet.	100% diet.	66% diet.	50% diet.
157·6 ± 2·2	137·3 ± 1·8	120·5 ± 2·4	150·5 ± 3·0	235·7 ± 5·4	233·3 ± 2·9

The figures obtained from the controls were similar to those from other normal mice (Bullough, 1949), but, as would be expected, those from the animals fed on restricted diets at 09.30 hours were subnormal. However, the evening fed animals of the 66 per cent and 50 per cent diet groups had abnormally high blood sugar levels, due doubtless to the meal which they had eaten some two or three hours earlier at the end of a fast period of at least 22 hours' duration.

From these results a general conclusion can be drawn that a reduction of diet to two-thirds or a half of what mice eat when fed *ad libitum* results in a lowered body weight, a reduced blood sugar level, and a depressed mitosis rate.

DISCUSSION.

It is evident that the mitosis depressing effects of starvation and of restricted diets are caused by shortages of key materials necessary to cell division. Previous work has indicated that by far the most important of these substances is carbohydrate, and the theory has been advanced that its function is to supply the energy requirements of mitotic activity by some process of glycolysis (Bullough, 1949). In comparison, the experiments of Tannenbaum (1947) have shown that restricted diets greatly reduce the genesis of all types of tumours, induced or spontaneous, and it is significant that once again it is the carbohydrate fraction of the food which has the greatest effect.

Theoretically it is possible that carbohydrate deficiency could prevent the appearance of tumours either by depressing the formation of the latent tumour cells, or by preventing or delaying the further growth of these cells, or by inhibiting or slowing the growth process itself. The first of these possibilities is dealt with in a following paper (Bielschowsky and Bullough, 1949), and it can be concluded at once that neither diet nor mitotic activity has any apparent effect on the formation of latent tumour cells by such substances as benzpyrene or methylcholanthrene. The last possibility may also be excluded, for Tannenbaum himself concludes that while drastic diet restrictions may retard tumour growth, this cannot be held to explain a reduction in tumour incidence.

It is the second possibility which is particularly interesting, since it is now believed that latent tumour cells do not grow unless they are stimulated to do so (Berenblum and Shubik, 1947). One of the most powerful stimulating, or developing, agents is croton oil, which induces hyperplasia. It is probable that any factor which induces hyperplasia can act as a developing agent for the latent

tumour cells simply because it increases the chances that any single cell, or group of cells, will receive the stimulus to multiply. In the normal body these developing factors probably act locally, although recent work has indicated, at least in the male mouse, that middle age is characterized by a generally raised mitosis rate which must itself assist in the development of any latent tumour cells which may be present. While the reason for the increased mitotic activity is still obscure, this observation may afford some explanation of the fact that middle age is characteristically the cancer age.

Since the development of latent tumour cells is assisted by conditions of hyperplasia, it is reasonable to suppose that conditions of hypoplasia will have the opposite effect. Hyperplasia increases the chances that the latent tumour cells will be stimulated to multiply, and so it results in the earlier development of tumours and in the appearance of many which would otherwise never have formed. Hypoplasia decreases the chances of stimulation, and so delays the development of those tumours which do form, and prevents altogether the appearance of many which otherwise would form. Thus it is possible to provide a logical explanation of Tannenbaum's results, and the fact that carbohydrate supply is the critical factor in both cell division and tumour genesis can be taken as corroborative evidence.

At last it is becoming possible to split the cancer problem into two separate parts: that which is concerned with the formation of latent tumour cells, and that which is concerned with their subsequent development. Perhaps the first problem is still a long way from solution, but it now appears possible that a practical control of cancer may be developed from a thorough understanding of those factors which govern normal cell division. If hypoplasia can be maintained without damage to health, as in effect Tannenbaum has shown that it can, then it may be a means of preventing tumour formation.

In conclusion, a comment is also possible on Tannenbaum's observation that mice kept on a reduced diet "appear younger, live longer on the average, and reveal fewer pathological changes in the tissues." This may well be related to the fact that hypoglycaemia not only reduces the rate of cell division of a host, but also that of a parasite. Hegner (1937) showed that the multiplication of the malarial parasite *Plasmodium cathemerium* is impeded in hypoglycaemic canaries, so that fewer birds become ill and fewer die. The converse is true in hyperglycaemic canaries, and Steinbach and Duca (1942) have also shown that hyperglycaemic rats, inoculated with bovine tubercle bacilli, develop larger and more numerous tubercles than do the controls.

SUMMARY.

Using Strong CBA males, it has been shown that starvation has a powerful effect in depressing epidermal mitotic activity, so that after 36 hours such activity is almost entirely eliminated. A similar effect is produced by restricted diets. Animals rationed to 66 per cent of what they would eat if fed *ad libitum* have an epidermal mitosis rate which is less than 40 per cent of that of well fed controls. If rationed to 50 per cent, the mitosis rate drops to about 15 per cent of that of the controls.

It is suggested that these observations provide an explanation of Tannenbaum's results on the prevention of tumour genesis by diet restriction. The induction

of hypoplasia by starvation decreases the chance that any latent tumour cells which may be present will receive the stimulus to multiply, and it is significant that in both cell division and tumour genesis carbohydrate supply appears to be the critical factor.

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EPIDERMAL MITOTIC ACTIVITY AND THE INDUCTION OF SKIN TUMOURS IN MICE.

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IN his last communication J. C. Mottram (1945) published the results of experiments designed to demonstrate a relationship between the tumour yield induced by benzpyrene and the number of epidermal mitoses present at the time of application of the carcinogen. Painting the skin of mice at midnight resulted in a higher yield of papillomata than did painting at midday. Mottram attributed this result to the diurnal variation in mitotic activity occurring in the skin, and he was of the opinion that the higher yield was due to the presence of an increased number of mitoses at midnight.

However, Mottram's belief that there are in fact more epidermal mitoses at midnight than at midday is not securely founded. There are many differing accounts of the diurnal mitosis cycle, and recently a double daily cycle has been described (Bullough, 1948*a*). It is now realized that the precise form of the cycle is determined by the routine of waking and sleeping, and this in turn may be varied from time to time by such factors as food, age, sex, condition, and laboratory routine (Bullough, 1948*b*). In the course of these investigations it was discovered that a more certain control of epidermal mitotic activity can be obtained through regulated starvation (Bullough, 1939), and this new technique has therefore been used to reinvestigate the problem raised by Mottram.

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MATERIAL AND METHODS.

At the beginning of the experiments all the mice were about three months old. In Experiments 1 and 2 they were males of mixed stock, and in Experiments 3 and 4 they were males and females of the Kreyberg's white label strain.

In Experiments 1 and 2 the mice were divided into groups of 20. The two groups A were deprived of all food, but not water, and 17 hours later they were given a single application of carcinogen, benzpyrene (Hoffman-La Roche) in Experiment 1 and methylcholanthrene (Eastman Kodak & Co.) in Experiment 2. In the benzpyrene experiment each animal received 0·1 ml. of a 0·3 per cent solution in acetone applied to the interscapular region of the skin, and in the methylcholanthrene experiments the dose was also 0·1 ml. of a 0·3 per cent solution in acetone. The mice were starved for a total period of 64 hours, after which they were fed *ad libitum*. Three days after the application of the carcinogen painting with croton oil (0·5 per cent in acetone) was commenced, and this was repeated twice weekly for 20 weeks, when the experiment was terminated. The mice of groups B received their food as usual both before and after the application of the carcinogen, and they were also painted with croton oil. During the third week of the experiment, when the mice had already been painted five times with croton oil, they too were starved for a total period of 64 hours. In Experiment 2 there was a third group, C. These mice were treated in the same way as those in the group B, except that the period of starvation was omitted altogether.

In Experiment 3, Group A contained 25 male mice which were deprived of all food 36 hours before each was painted with 0·05 ml. of a 0·3 per cent solution of benzpyrene in acetone. Thereafter, starvation was continued for a further 24 hours. The 25 male mice of group B were fed regularly both before and after the single application of the carcinogen. Four days later all the animals were painted with a 1 per cent solution of croton oil in acetone, and this was repeated three times a week for 20 weeks.

In Experiment 4, 30 female Kreyberg's mice were given massive doses of methylcholanthrene. At 15-minute intervals each mouse received four applications of carcinogen on the interscapular region, each application consisting of 0·1 ml. of a 0·6 per cent solution of methylcholanthrene in acetone. These animals were not starved and were not painted with croton oil. The experiment was terminated as before at 20 weeks.

RESULTS.

Each of the groups in Experiments 1 and 2 gave a tumour yield of about 40 per cent (Table I), most of the papillomata being discovered between the 12th and 15th weeks. This is in good agreement with the results obtained by Berenblum and Shubik (1947), who used a similar technique. No significant differences were found between the numbers of tumour-bearing animals in the starved and control groups, and it must be concluded that neither the state of nutrition nor the degree of mitotic activity are important in experiments of this kind.

In Experiment 3 only eight tumours appeared. It is probable that the potency of the croton oil was partly responsible for the low yield, and the smaller amount of benzpyrene must also have been a contributing factor. It will also be noticed that these papillomata appeared rather late.

TABLE I.—*The Tumour Yield from Mice which were Starved to Induce a Lowered Mitotic Activity at the Time of Application of the Carcinogen (Groups A) as compared with that from Well-fed Controls (Groups B and C).*

Experiment.	Treatment.	Number of mice used.	Survivors at time of first tumour.	Average latent period (weeks).	Number of mice with tumours.
1. A	Benzpyrene + croton oil	20	20	14½	9
1. B		20	19	14	8
2. A	Methylcholanthrene + croton oil	20	18	13½	7
2. B		20	19	13½	8
2. C		20	19	13	9
3. A	Benzpyrene + croton oil	25	25	16	4
3. B		25	25	20	4
4.	Methylcholanthrene (large dose)	30	30	10½	7

In Experiment 4, when one massive dose of methylcholanthrene was given to 30 mice, seven tumours appeared on the site of application during the subsequent 20 weeks, and some of them appeared as early as 5 or 6 weeks after painting. There were three carcinomata in this experiment, two of which showed their malignancy from the very beginning, while the third started as a papilloma and became malignant after a few weeks.

No malignant changes were observed in any of the mice which received a single small dose of benzpyrene or of methylcholanthrene, and which were subsequently painted with croton oil. All the papilloma-bearing mice of Experiment 2 were kept for further observation after the croton oil treatment had been stopped, but not a single carcinoma developed. On the contrary, a number of well-established papillomata disappeared during the subsequent months.*

DISCUSSION.

These results provide confirmation of the findings of Mottram (1944) and of Berenblum and Shubik (1947) that benign skin tumours can be obtained by the initiating action of a single small dose of a carcinogenic hydrocarbon, provided that croton oil is subsequently used as a promoting agent. However, contrary to the theory of Mottram, it appears that the final tumour yield is the same whether or not the mitotic activity of the skin is suppressed by starvation during the period of action of the carcinogen, and similarly Berenblum and Shubik have shown that a stimulation of mitotic activity during that period is without effect on the tumour yield. It follows that the differences observed by Mottram between the tumour incidence in mice painted at midday and in those painted at midnight cannot have been due to variations in the mitosis rates at these times and may well have occurred by chance.

Another interesting point emerging from the present results is that none of the papillomata obtained by means of small doses of benzpyrene or of methylcholanthrene progressed to malignancy, whereas three carcinomata were found among the 30 mice treated with a massive dose of methylcholanthrene. Thus it

* At the time of going to press it is necessary to add that, 5 months after the painting with croton oil, two formerly benign lesions became malignant.

seems that a single massive dose can set in motion a process differing from that induced by a small dose which is followed by repeated paintings with an unspecific irritant. Cramer and Stowell (1942*a*, *b* ; 1943) have given an excellent account of the great disturbance which follows treatment with large doses of methylcholanthrene. As after an overdose of X-rays, the damaged tissue seems unable to revert to its former state.

SUMMARY.

The application of a single small dose of a carcinogenic hydrocarbon to the skin of mice, followed by repeated doses of croton oil, leads to the formation of benign tumours. The yield of papillomata obtained in this way is independent of the number of mitoses present at the time of action of the carcinogen.

A single massive dose of methylcholanthrene results in the production of carcinomata as well as of papillomata, and it is suggested that the chronic damage inflicted by such treatment creates conditions which are especially favourable for the development of malignancy.

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PRIMARY PANCREATIC TUMOURS IN RATS FED P-DIMETHYLAMINOAZOBENZENE.

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PARA-DIMETHYLAMINOAZOBENZENE (pDA) is known to induce hepatic tumours when fed to, or injected into, rats or mice ; mice are the more resistant to the effect of the dye (Kinosita, 1937). Kinosita (1937) states that M. Tanaka succeeded in producing one transplantable sarcoma in a rat after several subcutaneous injections of large doses of pDA, but tumours in other situations have not been observed in animals receiving this compound. The rate of development of hepatic tumours due to feeding pDA can be modified by dietary factors. Some factors delay the appearance of tumours, others increase their incidence. Rusch, Baumann, Miller and Kline (1945) point out that, at that time, no dietary combination was known which prevented the eventual appearance of hepatic tumours if the dye was fed continuously for a long enough time. This group of workers adopted, as a routine, a procedure in which the rats receiving the azo-dye were examined by laparotomy after four months of feeding, and were killed after six months. They considered this method satisfactory for the estimation

of the effect of any dietary combination on the rate of development of hepatic tumours.

It has been reported (Hoch-Ligeti, 1946) that the addition of fresh milk daily to a rice diet containing 0·06 per cent pDA largely prevents the development of hepatic tumours even in animals receiving the dye continuously for more than 15 months. This protective action of milk has now been tested in the case of a semi-synthetic diet in which 17 per cent of the caloric value is supplied by protein and 30 per cent by fat. Ninety-five per cent of the rats fed on this semi-synthetic diet with the addition of 0·06 per cent pDA developed hepatic tumours after five months. The addition of fresh milk to this diet, containing pDA, again prevented the development of hepatic tumours even after 17 months of continuous feeding of the dye.

In three rats which failed to develop hepatic tumours after prolonged feeding with pDA, pancreatic tumours were observed; and since a similar occurrence has not previously been reported it seemed of interest to record this finding despite the small number of animals concerned.

EXPERIMENTAL.

All rats were of the Wistar strain, and about four months old at the beginning of the experiment. The basic diet for all animals consisted of starch 1260 g., sugar 630 g., casein 540 g., fat 420 g., cod-liver oil 7 g., mineral salt mixture (Glaxo) 126 g. The fat was a mixture of whale fat and palm oil. pDA was added in a concentration of 0·06 per cent. All rats received greens once weekly. The diet and water were given *ad libitum*.

Twenty of 50 rats receiving this diet served as controls, whilst the remaining 30 rats each received a daily addition of 10 ml. of fresh milk. The rats were weighed weekly and inspected for development of tumours. All rats grew at a normal rate.

RESULTS.

In Table I are summarized the findings of tumour localization and time of their appearance. The substitution of the mixture of whale fat and palm oil

TABLE I.—*Localization of Tumours in Control Rats and in Rats fed pDA.*

Number of rats.	Addition to the basic semi-synthetic diet.		Tumours.			
	pDA.	Milk.	In liver.	Time when observed (months).	In other organs.	Time when observed (months).
150	None	None	None	—	4 small intestine	4–6
20	0·06%	None	16	7–14	1 pancreas	15
					1 intestine	6
30	0·06%	10 ml./rat/day	1	19	2 pancreas	12, 14

for lard in a diet containing 0·06 per cent pDA delayed somewhat but did not prevent the development of hepatic tumours; the first tumours were palpable at the 7th month. Sixteen out of the 20 rats developed primary hepatic tumours during the 15 months of the experiment. As no tumours were palpable at the end of the 16th month in the rats receiving the milk supplement, laparotomy was done and the liver inspected. No macroscopical hepatic tumours were found.

Twenty-three out of the 30 rats of this group are still alive and without palpable tumours 20 months after the commencement of the experiment. Of the remaining animals one developed an hepatic tumour in the 19th month of the experiment, and four died between the 14th and 20th months without, however, developing tumours.

In two rats of this group primary tumours localized in the duodenal loop were found, one in the 12th month of the experiment and the other in the 14th month. A third tumour similarly situated developed after 15 months in a rat fed pDA but no milk ; this animal had failed to develop a hepatic tumour. The situation of these tumours suggested a pancreatic origin. They grew very rapidly, and considerable ascites developed in all three rats within a few days.

On microscopical examination, in two of the tumours (Rat CA 801 on the pDA control diet and Rat CL 917 receiving fresh milk) pancreatic tissue was recognized and the origin of the tumours was confirmed. Histologically these two tumours were very similar. They consisted of polyhedral cells in a state of rapid proliferation. Mitoses in all phases and pathological mitoses were numerous. Giant cells were found in many places. Metastases were widespread in the abdominal lymph glands and in the lymphatic vessels leading to the small intestine (Fig. 1–4).

In the liver of both rats there were many binucleated cells, patches in which the cells had a " foam cell " appearance and areas showing intense fatty degeneration. The adrenals, particularly from rat CA 801, showed an accumulation of brown pigment in the zona glomerulosa and small cysts in the cortex.

The third tumour (Rat CL 912, fed milk) appeared to be a carcino-sarcoma. No unquestionably pancreatic tissue was seen in the sections, but the anatomical localization of the tumour makes a pancreatic origin very likely. The tumour consisted mostly of young spindle-shaped cells, but epithelial cells concentrically arranged in distinct groups and many multi-nucleated cells could be discerned (Fig. 5, 6). This tumour resembled the description of certain tumours of hepatic origin produced by pDA in rats by Kinosita (1937). He describes these " extraordinary tumours " as being similar to reticulo-sarcomas or " composed of spindle-shaped cells and multi-nucleated giant cells. As another deviation some tumour cells, grouped in bulbous masses, show an evident tendency to concentric arrangement of cells which are more or less keratinized towards the centre similar to ordinary squamous cell epitheliomas." The liver of Rat CL 912 was infiltrated with tumour cells.

Over the two-year period in which the semi-synthetic diet was used, some 150 rats receiving this diet without the addition of pDA have been dissected. No pancreatic tumour was encountered. During this period carcinomas of the small intestine were found in three piebald and in one albino rat. In the present series of 50 rats fed pDA one similar intestinal carcinoma was found, in the 6th month of the experiment. As the spontaneous origin of this tumour cannot be excluded, it will not be considered here.

DISCUSSION.

The data presented indicate that the carcinogenic action of pDA is not exclusively on the liver but affects other organs as well. Its carcinogenic activity towards the liver however is much stronger, and the effect of the dye on other

organs can manifest itself only when the liver is either protected or resistant to pDA. Tumours not localized in the liver have not been observed previously, partly because of the preference of the compound for the liver and partly because experiments in which the liver has been protected were terminated too early for tumours to develop elsewhere.

Whether or not the substances in the diet which defer or prevent the production of hepatic tumours by feeding pDA are really anticarcinogenic or only protect the liver has been questioned. It seems that in the case of milk at any rate only the liver is protected from the carcinogenic activity of pDA, and that tumour development generally is not prevented. It is remarkable that the extra-hepatic tumours should arise in an intestinal gland, and this seems to suggest that organs which might be connected with the metabolism of pDA are attacked.

The conception that milk has a protective action on the liver only is interesting in view of the general plan of these experiments, the object of which was to find, if possible, an explanation of the prevalence of hepatic tumours in the natives in certain parts of Africa and Asia. It might be assumed that the effect of a carcinogenic factor which produces hepatic tumours, given a certain set of dietary habits and environmental conditions can be modified when the conditions differ. The observation that feeding pDA produces a large number of hepatic tumours on some diets, and that it provokes a certain number of tumours of a different localization when the liver is protected, might be helpful in explaining differences in the localization of tumours in different parts of the world.

SUMMARY.

In rats which did not develop liver tumours after receiving a diet containing pDA for 17 months, three pancreatic tumours were found; these occurred between the 12th and 15th months of the experiment. Two of the tumours were in rats in which the liver was protected by the addition of fresh milk to the diet.

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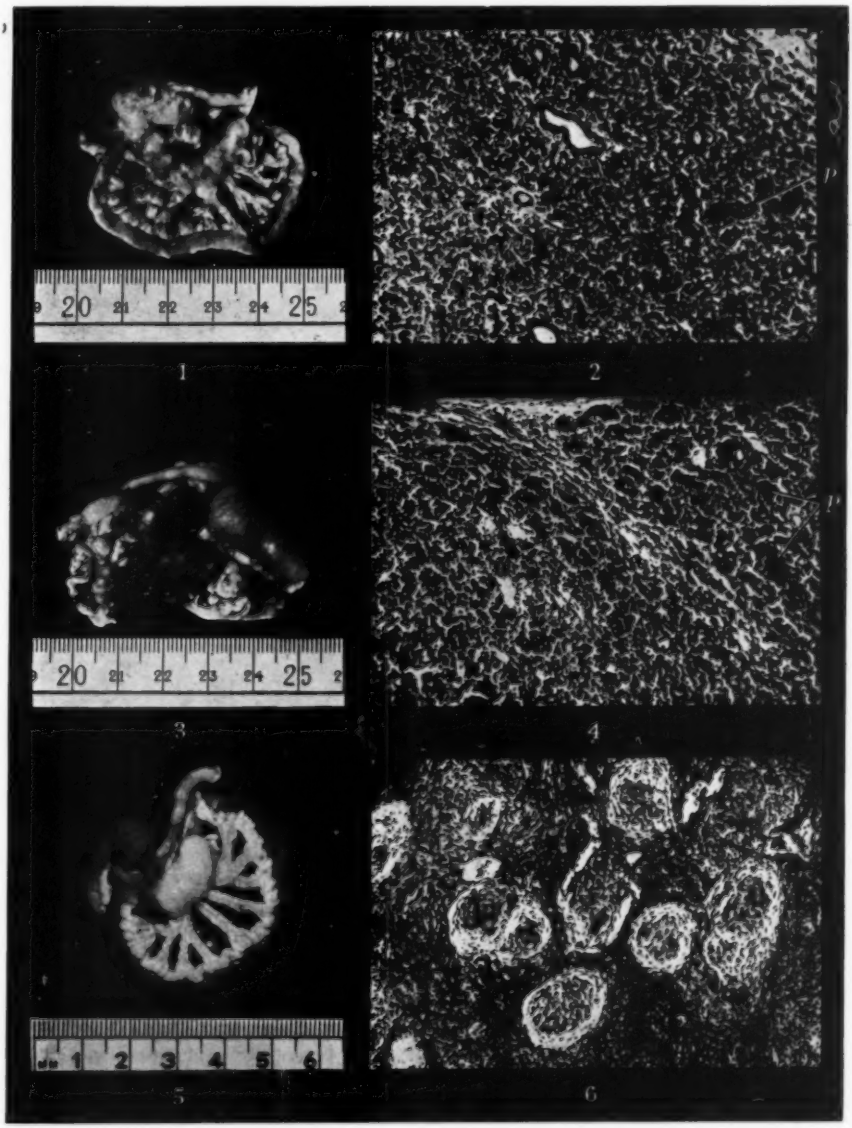
EXPLANATION OF PLATE.

FIG. 1 and 2.—Tumour from Rat CA 801. The rat was fed the basic diet containing 0·06 per cent pDA. Fig. 2 × 130. P = pancreatic tissue.

FIG. 3 and 4.—Tumour from Rat CL 917. The rat was fed the basic diet containing 0·06 per cent pDA and fresh milk. Fig. 4 × 85. P = pancreatic tissue.

FIG. 5 and 6.—Tumour from Rat CL 912. The rat was fed the basic diet containing 0·06 per cent pDA and fresh milk. Fig. 6 × 90.



Hoch-Ligeti.

ATTEMPTS TO INDUCE EPITHELIAL TUMOURS IN FOWLS.

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IT is a remarkable fact that while carcinogenic hydrocarbons readily induce sarcoma in the connective tissues of fowls, the same agents have not yet been shown to induce epithelial tumours in this species. Indeed until recently all attempts to induce epithelial tumours in fowls by any means seem to have failed.

Recently Gottschalk (1948) mentioned unsuccessful attempts to induce hepatoma in 89 fowls fed with " massive " doses of *p*-dimethylaminoazobenzene (DAAB), and maintained for over a year on a diet deficient in protein and riboflavine or enriched with biotin. He also quoted similar unsuccessful attempts to induce hepatoma with Butter Yellow by Kinosita (1940), and other failures to induce epithelial tumours in general with various carcinogens. One case of hepatoma was observed by Oberling, Guerin and Boic (1933) in a bird inoculated 9 months previously with a filtrate of Mill Hill 2 endothelioma. This hepatoma was not transmissible, but birds grafted with fragments of hepatoma developed leucosis which was transmissible. The aetiology of this hepatoma therefore is uncertain.

Induced epithelial tumours were amongst the teratomas obtained by Michalowsky (1929) following the injection of 5 per cent zinc chloride into the testicles of cocks.

Duran-Reynals (1946), while trying to transmit primitive renal tumours in fowls, obtained sarcomas but no epithelial tumours in the inoculated birds.

It may be that other workers have made extensive negative tests without recording them, but it seems advisable to describe our own mainly negative attempts, which have now extended over a period of about 17 years, and have yielded only two carcinomas, apparently caused by 2-acetylaminofluorene (2AAF).

In contrast with these negative results, Bielchowsky and Green (1945) obtained an anaplastic renal carcinoma in 1 of 5 Rhode Island Red cocks maintained on a mixed diet to which was added 2AAF, at the rate of 3–30 mg. per diem (total 5·5 g.) for 46 weeks. This bird was killed when moribund in the 87th week of the experiment, and the only other survivor was killed at the same time, in moribund condition, and found to have leukaemic enlargement of the liver and spleen. The other 3 birds died within the first year of experiment, without tumours.

Bielchowsky and Green also cite claims by Yamagiwa and Ohno (1918) to have induced carcinoma by injecting scarlet red and related azo compounds into fowls.

* Working under a full-time grant from the British Empire Cancer Campaign.

EXPERIMENTAL METHODS.

Skin painting and intramuscular injections.

(1) Barred Plymouth Rock (BPR) fowls were painted on the left side of the comb and injected into the breast muscle with the same sample of tar. No tumours of the comb resulted from this procedure, but sarcoma arose at the site of injection in 22 birds. These tumours have already been described (Peacock, 1933). Further details are given in Table I.

TABLE I.—31 *BPR Fowls, 2–3 Months Old.*

Date of first painting and injection with tar, September 29, 1931.

Year.	Deaths.	Total number of paintings.	Total number of injections.	Tumours.		
				Site of painting.	Site of injection.	Other sites.
1931	3	6	2–3	0	0	0
1932	14	27	4	0	10	1
1933	9	55	4	0	9	0
1934	4	65	4	0	1	0
1935	1	72	4	0	0	0
Total	31	72	4	0	20	1

One bird, No. 149, previously recorded as bearing a tumour, was found to have a necrobiotic lesion in the region of the encysted tar and a large haematoma in the breast muscle, but no sarcoma.

(2) Twenty-seven BPR fowls were painted on the right side of the comb with tar and injected in the right breast muscle with the same sample of tar.

While these experiments were running the first recognition of a pure hydrocarbon, 1:2:5:6-dibenzanthracene (DB), as a carcinogen was reported by Kennaway and Hieger (1930), and thereafter when the substance became available these birds were painted with a saturated solution of DB in chloroform instead of tar. In view of the lack of obvious reaction to the previous tar painting, light cautery of the comb with a hot platinum wire was applied to survivors immediately before painting with DB. At the same time the wattle was painted on the same side as the comb, to increase the area of skin exposed to the carcinogen. Further details of these experiments are shown in Table II.

TABLE II.—27 *BPR Fowls, 2–8 Months Old.*

Date of first painting and injection with tar, March 15, 1932.

Year.	Deaths.	Total painting with—		Total tar injections.	Tumours.		
		Tar.	DB.*		Site of painting.	Site of injections.	Other sites.
1932	7	17	0	2	0	2	1
1933	8	40	0	2	0	6	0
1934	5	50	7	2	0	4	1
1935	5	50	14†	2	0	4	1
1936	2	50	14†	2	0	1	0
Total	27	50	14	2	0	17	3

* DB—Dibenzanthracene. † Comb lightly cauterized before painting.

(3) Thirty-one BPR fowls were painted on the right side of the comb and wattle with a saturated solution of DB or BP (benzpyrene) in chloroform. These birds had previously been injected intramuscularly with DB in solution in lard in the right breast and with lard only in the left breast. The tumours arising at the sites of injection have already been described (Peacock, 1933, 1935). Further details of painting and negative results are given in Table III.

TABLE III.—31 *BPR Fowls, 3 Months Old, Painted on Comb and Wattle.*

Intramuscular injection 4 times in September–November, 1931, with 0·4 per cent dibenzanthracene in lard. Date of first painting with dibenzanthracene, June 4, 1934.

Year.	Deaths.	Total painting with—		Total injections.	Tumours.		
		DB.*	BP.*		Site of painting.	Site of injection.	Other sites.
1934	5	7	0	4	0	2	1
1935	1	10	0	4	0	1	0
1936	1	13	3	4	0	0	0
1937	1	18	5	4	0	0	0
1938	0	..	..	..	..	..	..
1939	1	18	5	4	0	0	0
Total	9	18	5	4	0	3	1

* DB—dibenzanthracene; BP—benzpyrene.

(4) Sixteen birds of various ages up to 2 years were similarly painted with a saturated solution of BP for a maximum of 42 paintings, and observed for periods up to 2½ years. No tumours were induced.

(5) Five BPR fowls, 1 year old, were painted with a saturated solution of 3:4-benzpyrene (BP) in chloroform on the right side of the comb 11 times at fortnightly intervals. These birds were injected intramuscularly with 1–5 ml. of their own whole blood on 9 occasions to control possible effects of local disturbance by a presumably non-carcinogenic inoculum as a check on the tar injection in previous experiments. Three birds died within 1 year of the start of the experiment, 1 after 2½ years and 1 after 4½ years. These birds developed no tumours at any site.

(6) Five Brown Leghorn (BL) fowls and 1 White Leghorn (WL), 2 years old, were painted 10 times at weekly intervals on the right side of the comb with a 0·3 per cent solution in acetone of 9:10-dimethyl-1:2-benzanthracene (DMB). Two of these birds had been injected a year previously, one in the breast muscle, another in the right wattle, with 5 ml. and 1 ml. respectively of a saturated aqueous solution of "styryl 430" without obvious effect. No tumours developed.

Skin painting and implants into the preening glands.

(7) Twenty BL pullets, 3 months old, were painted 84 times at weekly intervals with a 1 per cent solution of DMB in acetone on the under-surface of the right wing, and with a 1 per cent solution of the same substance in benzene on the corresponding aspect of the left wing. Five died within 6 months of the start of the experiment. Nine survivors were painted in the neighbourhood of the preening gland with the acetone solution and 6 with benzene solution of 1 per

cent DMB. The only survivor after 29 months had a 25 mg. pellet of 3:4:5:6-dibenzcarbazole (DBC) implanted into the right preening gland, followed by twice-weekly painting of the preening gland area with 0·1 per cent solution of croton oil in acetone. At the time of writing this bird is alive and well, without clinical signs of tumour, 3 years after the start of the experiment. No tumours occurred in other birds in this experiment.

(8) In preliminary experiments, 7 BL fowls were injected into the preening gland with solutions of DMB in arachis oil. The fluorescent solution was expelled through the duct of the preening gland and, as there was no evidence of retention in the gland cavity after a few days, this type of experiment was abandoned.

Implants into the preening glands.

Three groups of 6 BL hens had the following pellets implanted into the left preening gland :

15–30 mg. pellets of methylcholanthrene.
30 mg. pellets of N:N-dimethylaminostilbene.
23 mg. pellets of dibenzanthracene.

A fourth group of 7 BL hens had 15 mg. pellets of dibenzcarbazole implanted into the left preening gland.

Starting a week after the implantation operation, these fowls were painted over the preening gland twice weekly with a 1 per cent solution of croton oil in acetone.

Following such implantations of pellets the preening oil from the implanted side showed blue-violet fluorescence for variable periods up to 6 months, as compared with the yellow fluorescence of the oil from the opposite control gland.

Apart from slight squamous metaplasia of the epithelium of the preening gland in birds implanted with N:N-dimethylaminostilbene, no pathological changes have been seen in the preening glands of these birds. These experiments are not yet complete, but some 20 birds have survived for 18 months without developing tumours.

Intramuscular injection of orthoaminoazotoluene.

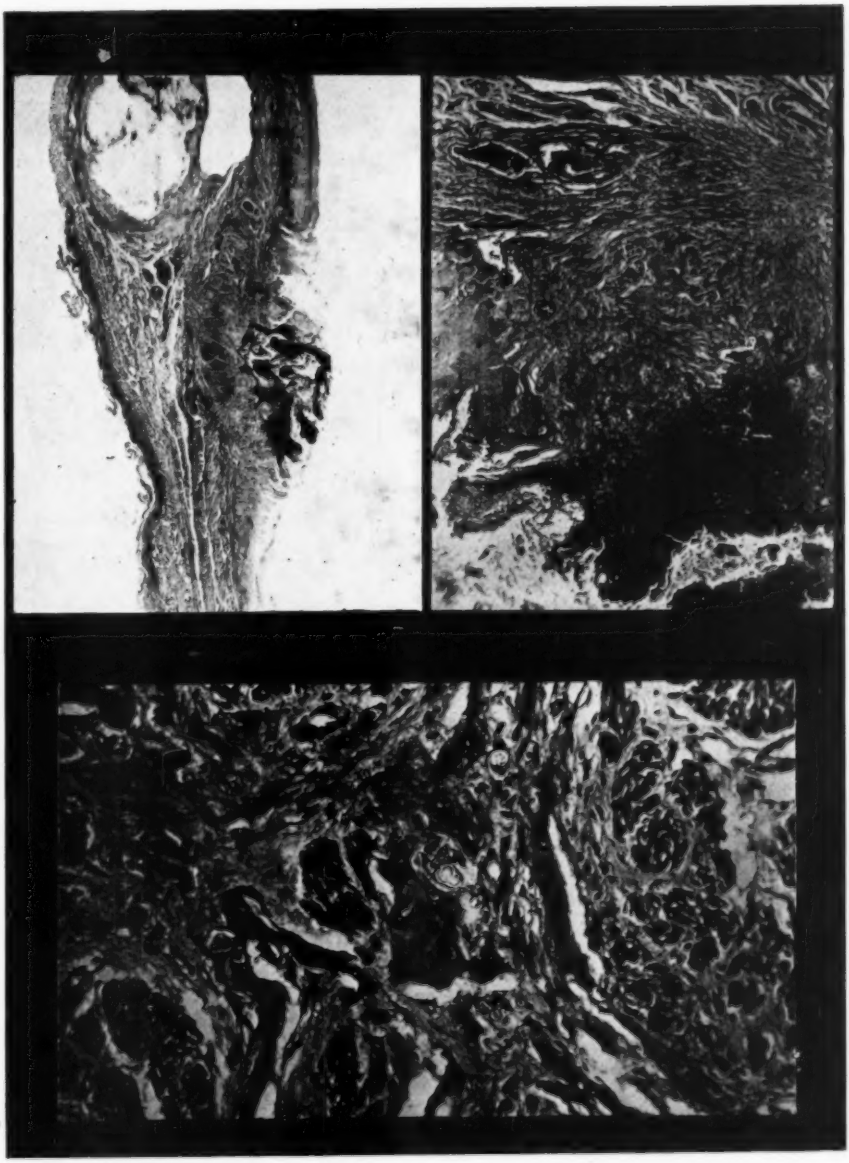
(10) Ten BL hens were injected in the pectoral muscles with 0·5 to 1·5 ml. of a 5 per cent solution of orthoaminoazotoluene in lard. Most birds died within 2 years, and no tumours were seen in the liver or at the site of injection.

Injections into the crop.

(11) Six BL hens, 3 BL cocks and 3 WL cocks were each injected into the crop twice a week with 2·5 ml. of a 1 per cent solution in arachis oil of 2AAF. Of 7 survivors for 14 months, a WL cock was killed because it had acute inflammation of the lachrymal glands with copious exudate. Post-mortem examination revealed a localized tumour in the mucosa of the crop ; this proved to be an early ulcerated squamous carcinoma (Fig. 1–3). This bird had received a total of

EXPLANATION OF PLATE.

FIG. 1.—Ulcerated lesion in crop of fowl repeatedly injected with 2-acetylaminofluorene. × 6.
FIG. 2.—Portion of base of ulcer shown in Fig. 1. Normal epithelium on extreme left of section. Ulcerated squamous carcinoma invading muscular wall of crop. × 32.
FIG. 3.—Squamous carcinoma invading muscle. × 160.



1 2

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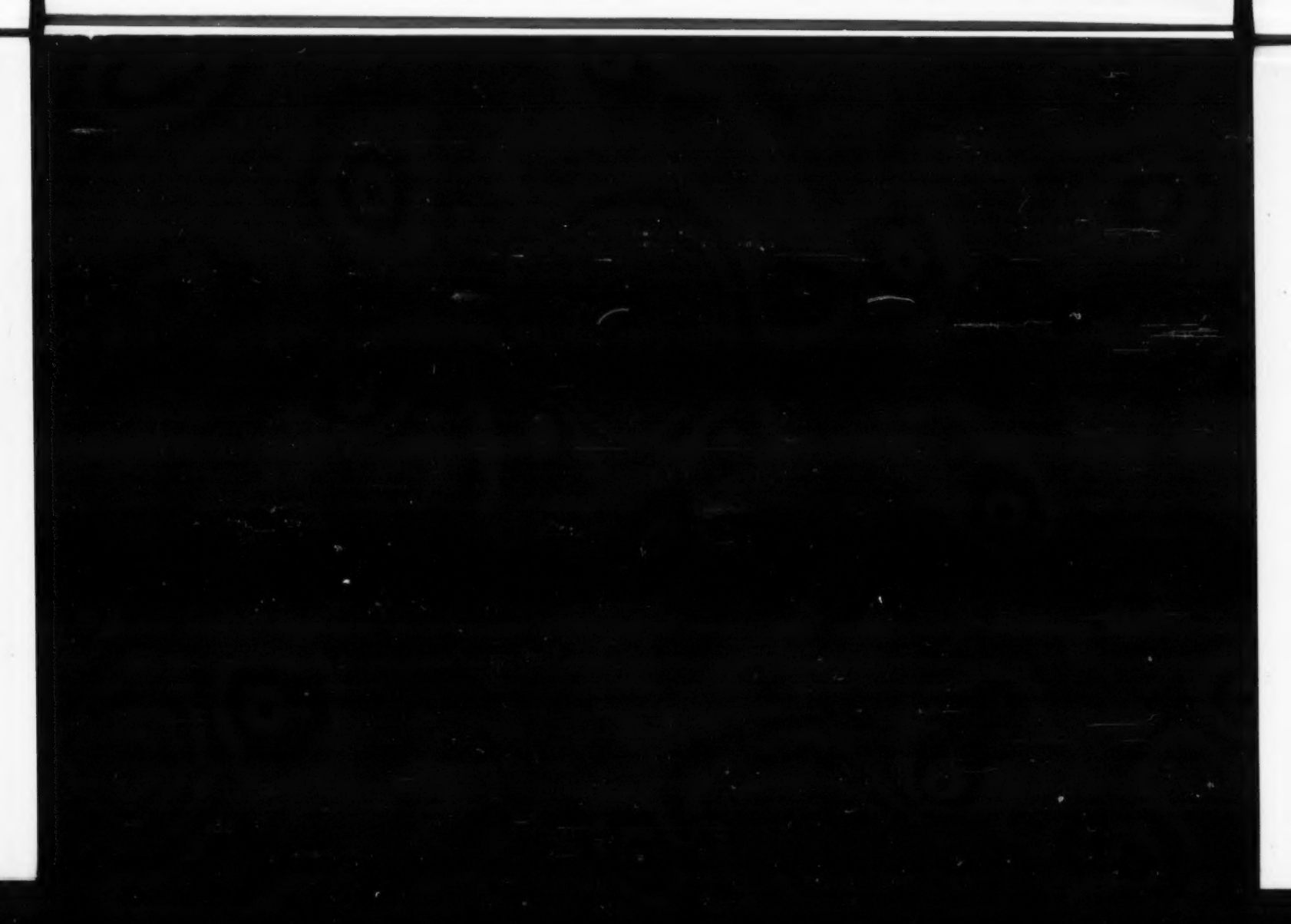
Peacock and Peacock.



3·1 g. 2AAF by injection into the crop. It had also had a pellet of 22 mg. methylcholanthrene implanted in the right preening gland 14 months previously, without causing any gross pathological changes. The other birds had all been implanted with similar pellets of methylcholanthrene, N:N-dimethylaminostilbene, or 1:2:5:6-dibenzanthracene into the right preening gland, without obvious effect.

Of the remaining 6 survivors after 17 months, 2 had palpable nodules in the wall of the crop. One of these, a BL hen, was killed because it was rapidly losing weight and was found to have several ulcers in the crop varying in diameter from about 1 to 8 mm., and partly covered by tenaceous slough. There were also numerous oily cysts in the connective tissues around the crop and in the muscular and submucous tissues of the crop itself representing remnants of previous injections of 2AAF in arachis oil. The remainder of the alimentary tract was thoroughly examined but no other lesions were found.

The liver and spleen were small and paler than normal but showed no localized lesions. The kidneys were pale but showed no gross lesions. Other viscera showed no gross abnormality. The ovary was in a resting stage and the oviduct was involuted.

Sections of the ulcers in the crop show that it was the seat of at least 3 apparently separate squamous carcinomas. A biopsy 3 months before death had shown no malignant growth. This bird had had, in all, 48 injections of 1 per cent 2AAF in arachis oil, and 32 injections of 1 per cent 2AAF in 16 per cent alcohol in water containing 0·3 per cent gelatin, equivalent to a total of 4·15 g. 2AAF. 0·25 ml. 9:10-dimethyl-1:2-benzanthracene in arachis oil was injected in the left preening gland (see para. 8 above) in September, 1945.

Injections of embryo-epithelium plus carcinogen.

(12) Ten-day chick embryo epithelium plus a few crystals of DB injected into 1 WL cock and 1 WL hen have given rise to no tumours up to 18 months after injection. Ten-day embryo epithelium plus pellets of 5–30 mg. of methylcholanthrene were injected into the pectoral muscle of 1 WL cock and 19 WL hens. No tumours have developed. Three birds were killed 12 months after injection, and the pellets were found encapsulated with no evidence that absorption had occurred.

DISCUSSION.

In view of Bielschowsky's observation of a renal adenocarcinoma in a bird fed with 2AAF and of our cases of squamous carcinoma in birds injected into the crop with this substance, further tests are being carried out. All other substances tested by us have failed to induce any epithelial tumour in the fowl.

Lacassagne and Latarjet's (1946) experiments with mice irradiated with ultraviolet and subsequently painted with methylcholanthrene showed that where the sebaceous follicles were destroyed before methylcholanthrene painting, tumours occurred only in the unirradiated skin. This observation suggests a possible explanation for the complete failure of local application of carcinogenic hydrocarbons to induce squamous carcinoma in the skin of fowls, which have no sebaceous follicles. It was this consideration that led us to try implantation of pellets of carcinogens into the preening gland of fowls.

The histology of the preening gland resembles that of the mammalian sebaceous gland, though the grouping of many glands together opening into a common

cavity is characteristic of birds. N:N-dimethylaminostilbene was kindly given by Prof. Haddow, who first described the carcinogenic properties of this substance, a peculiar feature of which is that, like 2AAF, it tends to induce tumours of the external auditory meatus in the rat.

Examination of the external meatus of rats some hours after intraperitoneal injection of a solution of N:N-dimethylaminostilbene showed an increased fluorescence as compared with controls. It was not found possible to obtain spectrographic evidence of the nature of the fluorescent substance in rats' ears. It was thought that excretion of fat-soluble carcinogens might be found in the preening oil of birds, as appreciable amounts of secretion can be collected by squeezing the glands.

No increased fluorescence of the preening oil was found, however, after injecting birds intramuscularly with N:N-dimethylaminostilbene.

Direct implantation of potent chemical carcinogens has failed to induce malignant changes in the preening glands of 37 birds as already stated. It may be that essential differences in the metabolism of different species make apparently similar tissues resistant in one and susceptible in another.

Comparisons between the incidence of tumours in different species as compared with man may be misleading, because most domestic animals are killed as soon as their economic value begins to fail. The low incidence of carcinoma in fowls might be explained on these grounds.

On the other hand, mammals differ in their susceptibility to the action of carcinogens. The apparent resistance of the skin of rats to carcinogenic tar was overcome by Watson (1931) by washing the rats' skin with fat solvents before tarring. Passey (1938) kept dogs for many years after painting their skin with tar, and though he observed melanoma in 2 out of 9 animals after more than 6 years of experiment, no case of squamous carcinoma occurred.

Recently, Pfeiffer and Allen (1948) described determined attempts to induce tumours in 50 Rhesus monkeys, by painting or injecting them with methylcholanthrene, DB or BP. All such attempts have failed, though the animals survived for periods up to 10 years, or approximately two-thirds of their expectation of life. That monkeys are not immune from squamous carcinoma is evident from the reports recently published of 3 cases of spontaneous squamous carcinoma of the oral cavity in monkeys(Klüver and Brunschwig, 1947 ; Zuckerman, 1930).

Spontaneous tumours, mostly of mesodermal origin, occur frequently in fowls, though no statistics comparable with human mortality rates are available because most fowls are killed in early adult life. Greenwood, Blyth and Carr (1948) recently discussed some of the factors influencing tumour mortality in fowls, and contrast the high cancer death rate, 19·5 per cent of autopsies reported by Goss (1940), with a death rate of 7·4 per cent in the Edinburgh inbred " low cancer " flock of Brown Leghorns. In both flocks all birds dying were examined post mortem.

The histogenesis of spontaneous fowl tumours was considered by Olsen and Bullis (1942). About 50 per cent of the tumours in their series were some form of leucosis, and epithelial tumours were rare (30 carcinomas amongst 216 spontaneous malignant tumours). Carcinoma of the ovary and embryonal nephroma were the most frequent spontaneous epithelial tumours. Only 1 case of epithelioblastoma of the skin occurred in their series.

Feldman (1932), in reviewing the literature, records 2 cases of squamous carcinoma of the floor of the mouth in fowls, and 2 carcinomas of the skin of the neck. A case of keratinizing squamous carcinoma of the buccal mucosa in a fowl was described and well illustrated by Puente-Duany (1932).

The only case of spontaneous squamous carcinoma seen by us in over 3000 post-mortem examinations of fowls, over a period of 20 years, occurred in a BPR fowl that had been unsuccessfully grafted with fragments from a chemically-induced sarcoma. The bird died with a tumour of the thymus, showing pleomorphic characters, and probably having arisen from multiple points of origin. Part of the tumour was anaplastic, but parts showed the characters of squamous carcinoma.

It seems that the skin of fowls is peculiarly resistant to the action of carcinogens, but the reason for this resistance remains obscure.

SUMMARY.

1. Protracted attempts to induce epithelial tumours in the skin of fowls by means which have proved effective in rodents have failed.

2. The absence of sebaceous follicles in fowls and the concentration of the sebaceous glands in the paired preening gland prompted us to implant potent carcinogens into the cavity of these glands. No tumours and only slight squamous metaplasia have followed this procedure during 2 years' observation.

3. Repeated injection of 2-acetylaminofluorene into the crop has been followed in two out of 5 surviving birds by ulcerated squamous carcinoma of the wall of the crop, 14 months and 21 months respectively after the start of the experiment.

We should like to thank Dr. Edward Duffy for kindly taking the low-power photomicrograph.

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THE INFLUENCE OF SOLVENTS ON TISSUE RESPONSE TO CARCINOGENIC HYDROCARBONS.

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THE need for controlling the influence of solvents used as vehicles for carcinogenic hydrocarbons was recognized by Burrows, Hieger and Kennaway (1932), who reported experiments designed to test the fat solvents then in use for this purpose, for possible carcinogenic action on the connective tissues of rats and mice. Subsequent work showed that in fact lard, which had been previously heated to 140° C., was a potential carcinogen for the connective tissues of fowls (Peacock, 1933), and that lard alone could give rise to spindle-cell tumours in rats (Barry and Cook, 1934); and later that lard, olive oil and other fatty materials could induce connective-tissue tumours, some of which appeared to be malignant in rats, but not in mice, in the experience of Burrows, Hieger and Kennaway (1936).

The first instance of undoubted sarcoma induction at the site of the injection of lard in fowls cited above was complicated by the fact that the same birds had been injected with a solution of 1:2:5:6-dibenzanthracene in lard in the right breast and with lard alone in the left breast. Tumours developed in the right breast only in 7 of these birds, and in both breasts in 3 others. The lard had been heated to 140° C. during the preparation of the material, and cooled to 40° before injection. The following comment is quoted from the above reference: "The possibility that heated lard alone may have determined the occurrence of tumours in a susceptible bird cannot be excluded. . . . Incidentally these experiments suggest that the quantity of dibenzanthracene actually required to initiate sarcomatous growth must be remarkably small, since the bulk of the the injection appears to remain encapsulated throughout the experiment" (Peacock, 1933). The latter assumption subsequently proved to be wrong. To test this point, Chalmers (1934) analysed the contents of encapsulated matter from the site of dibenzanthracene lard injection in one of Peacock's sarcoma-bearing fowls, which died 7 months after the last injection. The method of spectrographic analysis employed was capable of detecting 0·01 mg. of dibenzanthracene in an extract of fowl muscle, but failed to detect dibenzanthracene in the extract of encapsulated debris from this bird.

Further tests on birds in the same experiment confirmed the absence of demonstrable dibenzanthracene 6 months or more after injection. In order to avoid the use of foreign fats, chicken fat and egg yolk fat were used as vehicles for the injection of dibenzanthracene into fowls. It was found that when either

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of these solvents was used as vehicle dibenzanthracene rapidly disappeared from the site of injection, and was undetectable after 3 weeks. In parallel experiments (Peacock, 1935, 1936*a*) similarly injected birds were kept for longer periods, to test the influence of the solvent on the carcinogenic response. No tumours occurred in 12 birds injected with chicken-fat containing 1–2 mg. dibenzanthracene in 0·4 per cent solution. Moreover, in this group no tissue reaction could be found at the site of injection after a few months, indicating that the homologous fat had caused little disturbance. However, with egg yolk fat as vehicle for dibenzanthracene in 0·4 per cent solution, 2 metastasising sarcomas occurred among 4 birds that survived 9 months or more after injection. Birds injected with yolk fat showed fibrous cysts at the site of injection, similar to those observed in the lard experiment, and in contrast with the absence of such local reaction in birds injected with chicken-fat. There seemed, therefore, to be some aetiological connection between the "foreignness" of the solvent used as vehicle and the local tissue reaction and the ultimate carcinogenic response to dibenzanthracene.

Owing to the costly and time-consuming nature of long-term experiments on fowls work was begun on similar lines on mice. While these experiments were in progress Berenblum (1938) reported the co-carcinogenic action of croton oil. He had previously shown (1929) that mustard gas in dilute solution and (1935) cantharidin had anticarcinogenic properties for the skin of mice, and in the interim had found that no simple relationship between irritant and co- or anti-carcinogenic action was demonstrable. More recently Friedwald and Rous (1944*a*, *b*) reported that chloroform and turpentine both promote the carcinogenic action of methylcholanthrene, and Mottram (1944*a*, *b*) demonstrated that repeated painting with croton oil or resin could elicit a carcinogenic response in mice painted only once with 3:4-benzpyrene.

Thus it is now obvious that solvents may have co- or anti-carcinogenic influence in addition to their capacity as solvents to retain chemical carcinogens at the site of injection for variable periods.

On the basis of the lack of reaction in fowls to injections of chicken fat, we used mouse-fat as a solvent for benzpyrene injected into mice, with the idea of exciting a minimal disturbance due to the solvent. In this species also we observed very little tissue reaction to homologous fat. It seemed likely, therefore, that benzpyrene dissolved in mouse-fat would be readily absorbed along with the fat from the site of injection, and this proved to be the case. In a series of experiments in which various solvents were used as vehicles for benzpyrene it was found that those solvents which remained encapsulated at the site of injection, e.g. olive oil, tended to retain the dissolved benzpyrene as judged by the persistence of violet fluorescence, whereas in the case of those that were rapidly absorbed, e.g. mouse fat or ether, fluorescence could not be detected after a few weeks (Peacock and Beck, 1938). Moreover, in the former group the incidence of sarcomas was high, and in the latter group it was low. This suggested that there may be an optimal period for the retention of a carcinogen at the site of injection for the induction of a tumour, and that in mice this period was about 5 months.

Dickens and Weil-Malherbe (1942) at first confirmed our observation with mouse fat, but later (1946*a*), as a result of intensive experiments, found that the effect was not constant and was related to the phospho-lipid content of the

solvent. Fats with a high phospho-lipid content seemed to inhibit carcinogenic action, while those with a high cholesterol content seemed to enhance it. To obviate variations between samples of fat of animal or vegetable origin, Dickens and Weil-Malherbe adopted tricaprylin as a solvent for benzpyrene in further experiments. They also reported the interesting fact that certain purine compounds are good solvents for hydrocarbons giving aqueous solutions (Weil-Malherbe, 1946*a*).

It had already been shown by Andervont and Lorenz (1937) that serum was a solvent for benzpyrene and other hydrocarbons.

The solvent capacity of the circulating blood must thus be a factor in determining the localization in, or elimination of hydrocarbons from, the site of their injection; but there is, as Dickens pointed out (1947), a distinction to be made between elimination of unchanged hydrocarbon and local metabolism, processes which might elicit very different local tissue reactions, though either would reduce the amount of detectable hydrocarbon at the site of injection.

It still remained to be shown whether or not the fluorescence at the site of injection of benzpyrene (Peacock and Beck, 1938) throughout the latent period of carcinogenesis was due to the unchanged hydrocarbon. In the experiment now reported tricaprylin was used as vehicle for the injection of benzpyrene, as advocated by Dickens and Weil-Malherbe.

EXPERIMENTAL.

Thirty-six mice of mixed stock were each injected subcutaneously in the right flank with 0·3 ml. of a 0·1 per cent solution of benzpyrene in tricaprylin; 3:4-benzpyrene was supplied by Ward, Blenkinsop & Co., Ltd., London, and tricaprylin by British Drug Houses, Ltd. The mice were killed after different periods (Table I), the subcutaneous tissues exposed and examined for ultra-violet fluorescence. Sixteen of 20 mice killed at various times over a period of 7 months had fluorescent material located at the original site of injection; ulceration and sepsis at the site of injection killed 4 mice early in the experiment. In 3 of these mice no sign of the injected material was found, probably due to sloughing and leakage from the site of injection. The fourth had a small spot of fluorescence. These mice were not examined further; in the remainder the fluorescent material was extracted with benzene, with the exception of 2 mice, which died after $4\frac{1}{2}$ and $5\frac{1}{2}$ months, both showing persistent fluorescence and tumours at the site of injection.

After the third month of experiment tumours began to be clinically recognized at the sites of injection. As most of these tumours were similar to many such induced sarcomas in mice, not all were histologically examined. One tumour in the abdominal cavity associated with intraperitoneal deposits of fluorescent material was probably induced by benzpyrene injected accidentally through the abdominal wall. This tumour was a pleomorphic sarcoma. Another tumour occurred in an ulcerated area at the site of injection. This proved to be a squamous carcinoma. Of the remaining tumours the only one examined histologically was the familiar type of spindle-cell sarcoma.

As far as the injected benzpyrene was concerned the gross appearances in normal and ultraviolet light varied considerably in different mice, and the preliminary method of extraction was modified to suit the local condition. In most cases one or more small fluorescent cysts were present, and these were opened and

their contents expressed into 2 ml. benzene, yielding a fluorescent solution. In other mice numerous minute spots of brightly fluorescent material pervading a tumour or merely a general fluorescence localized in the neighbourhood of the site of injection were found. The region of tissue bearing the fluorescent material was dissected out in order to minimize the extraction of normal or adventitious fluorescent material which might mask the fluorescence of small traces of the carcinogen. In these cases the fluorescent tissues were triturated in benzene with a glass rod, or if this was unsuccessful the tissue was hydrolysed with 10 per cent aqueous alcoholic potash (50 ml. EtOH to 50 ml. H_2O), the alcohol removed, the unsaponifiable fraction extracted with 5 ml. benzene, and the benzene extract dried over anhydrous sodium sulphate.

Since the main object of these experiments was to demonstrate the presence or absence of benzpyrene after various periods, the experiments were not conducted quantitatively. However, the limit of sensitivity of the method of detection provides some indication of the quantities involved (*vide infra*). To eliminate the possibility of a false positive result due to traces of benzpyrene adhering to the apparatus used in the experiments, benzene washings of each piece of chemical apparatus or other instrument used, were examined under the ultraviolet lamp immediately before its use. Attention was drawn to the necessity for such precautions recently (Anderson, 1947*a*). Owing to the difficulty of eliminating minute traces of material from apparatus, such traces may be recorded by the fluorescence method of detection, and lead to a false positive result. The benzene (British Drug Houses, Ltd., AR.) used was rectified before use and was free from visible fluorescence. It is considered that the precautions employed exclude the possibility of any false positive result.

The fluorescence spectra were recorded with a Hilger Medium Quartz Spectrograph, using Ilford Iso-Zenith Plates. A small glass cell of 0·5 ml. capacity held the solution for test close to the slit of the spectrograph, and the fluorescence was excited by radiation from a G.E.C. Osira lamp focused on to the cell by a condensing lens. The slit width used throughout was 0·1 mm. and the exposures varied from 1–60 minutes, depending on the fluorescence intensity of the solution.

RESULTS.

The nature of the fluorescent material at the site of injection.

The relative intensities of the characteristic bands of the fluorescence spectrum of benzpyrene in pure benzene (Fig. 1, Spectrum (*a*)) are dependent on the sensitivity of the photographic emulsion to the different wavelengths. With the apparatus used in these experiments it is possible to obtain a good spectrum of benzpyrene at a concentration of 0·001 per cent with an exposure of 5 minutes, while if a 60 minutes' exposure is used it is just possible to detect the two strong groups of bands at 4030–4120Å and 4260–4400Å at a concentration of 1 μg./100 ml. This represents the practical limit of the technique, for it is just possible to detect fluorescence in a solution of this concentration by visual inspection. The volume of solution used in the test is 0·5 ml., representing 0·005 μg. benzpyrene at the limiting concentration.

Detection of benzpyrene in tissues examined by the technique described above implies that at least 0·02 μg. benzpyrene was present. In fact, however, the quantities of benzpyrene present at the sites of injection must have greatly

exceeded this value for several reasons: (1) No attempt was made to extract all the fluorescent material present at the site of injection. (2) The exposures required to demonstrate benzpyrene were under 60 minutes in every case. (3)

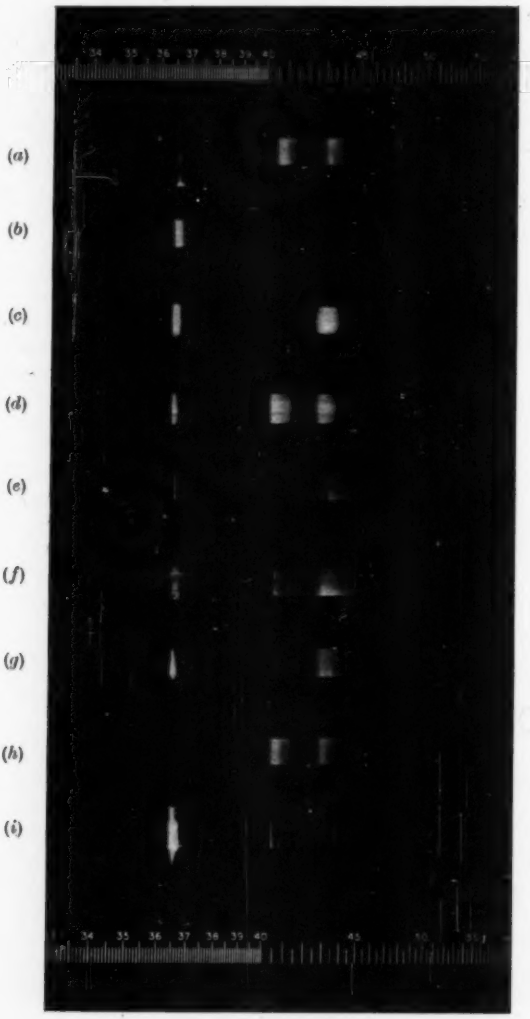

FIG. 1.—Fluorescence spectra of 3:4-benzpyrene and metabolites.

In all these cases the benzpyrene bands were very much stronger than those obtained with a solution of concentration 1 μg./100 ml. benzpyrene (limit of sensitivity of the method).

In long exposures reflection of mercury lines is recorded along with the fluorescence spectrum of the extract under examination. This is not a disadvantage, as it is possible to distinguish between the sharp lines of the mercury spectrum and the characteristic groups of bands due to the fluorescence of benzpyrene. The mercury lines accurately check the wave-length scale.

Spectrum (*b*) shows the weak mercury line at 4040 Å and 4070 Å in approximately the same region as the strong fluorescent bands of benzpyrene at 4030–4120 Å. The strong mercury line 3650 Å stands out clearly in the near-ultraviolet region ; the latter line can be seen in all fluorescent spectra taken with this equipment, but the weaker mercury lines in the visible spectrum are only obvious after long exposures.

Spectrum (*c*) exposure 20 minutes shows the fluorescence of a metabolite of benzpyrene extracted from the faeces of rats fed with benzpyrene dissolved in milk, and corresponds to the fluorescent derivative of benzpyrene detected by Peacock (1936*b*) in the bile of rabbits, guinea-pigs and fowls after intravenous injection of benzpyrene colloid. It was later deduced that this derivative was a monohydroxy benzpyrene (Chalmers and Crowfoot, 1941), and further work indicated that it was 8-hydroxy-benzpyrene (Berenblum, Crowfoot, Holiday and Schoental, 1943). Weigert and Mottram (1946) have pointed out that the formation of a monohydroxy benzpyrene may be preceded by the production of a diol, and have published fluorescence spectrograms of fractions extracted from tissues exposed to benzpyrene in which the main fluorescence is in the region 4200–4600 Å. Weigert, Calcutt and Powell (1947) showed that in mouse skin painted with benzpyrene the only detectable metabolite over periods up to 24 hours is that corresponding to Weigert and Mottram's BPX_2 represented by them by the following formula :

where R_1 and R_2 are unidentified cell constituents.

The different fluorescence spectra encountered in the present investigation are now considered in relation to the length of time after injection.

(1) *Extracts made 4 months after injection.*—Fluorescent material was extracted from 10 mice. A suitable spectrum was obtained from all of these, with exposures of 5–10 minutes—a representative spectrum is reproduced as Spectrum (*d*). All the spectra were the same, and are identical with that of benzpyrene (Spectrum (*a*)).

(2) *Extracts 5 months after injection.*—Fluorescent material was extracted from 2 mice. Spectra (*e*) and (*f*) correspond to these extracts. Both spectra have the benzpyrene bands at 4030–4120 Å, but the fluorescence also extends over the region 4260–4600 Å, which coincides with the longer wave-length region of the benzpyrene spectrum and also with the range of the benzpyrene metabolites. The interesting feature of these spectra is that they show an increase in the intensity of the fluorescence in the latter region, compared with the spectrum of unchanged benzpyrene. These spectra are interpreted as a mixture of the spectra of benzpyrene and another material with a spectrum similar to that of the fluorescent metabolites of benzpyrene.

(3) *Extracts 6 months after injection.*—Fluorescent material was extracted from 2 mice. The first of these had a tumour in which was embedded a small blue fluorescent cyst. Spectrum (*g*) was derived from material extracted from this cyst (20 minutes' exposure). It is similar to those of extracts from the mice killed at 5 months. The other mouse had 2 cysts with pink fluorescent pus and a small bluish fluorescent cyst. The corresponding spectrum (*i*) (60 minutes' exposure) shows none of the bands of benzpyrene. It is considered probable that the material from this cyst at the site of injection is a metabolite of benzpyrene. The spectrum consists of a band from 4300–4500 Å. There was not sufficient material for a detailed study of the chemical properties of the extract, but it was found that the fluorescence was unaffected by alkali. The available evidence strongly suggests that this material is similar to the known metabolites of benzpyrene. Spectrum (*h*) is that of benzpyrene. It will be seen that the super-position of Spectra (*h*) and (*i*) would give a spectrum similar to (*e*), (*f*) or (*g*), and it is suggested that these spectra are to be explained in this way as mixtures of benzpyrene and one or more of its metabolites.

(4) *Extracts 7 months after injection.*—Fluorescent material was extracted from 2 mice, both bearing tumours, one large and one small. The extract from the mouse with the large tumour gave a spectrum similar to that shown in Spectrum (*i*) after 60 minutes' exposure. There was no evidence of the presence of benzpyrene. The fluorescence spectrum of the extract from the mouse with the small tumour showed neither benzpyrene nor a known metabolite, but gave merely a continuous spectrum.

Table I shows the relationship which exists between the tumour incidence and the persistence of benzpyrene at the site of injection. It may be noted that the 10 mice which survived longest had no tumours and no persistent fluorescence at the site of injection.

TABLE I.

Duration of experiment. (days.)	Number of mice killed.	Tumour incidence.	Fluorescence of site of injection.	Spectrographic examination.
123	12	3/12	10/12	Benzpyrene identified in the 10 fluorescent extracts, including tumour mice.
153	3	1/3	2/3	Benzpyrene in 2 fluorescent extracts, including tumour mouse ; additional band suggests metabolite in both.
198	2	1/2	1/2	Benzpyrene in tumour mouse. ? Metabolite in other mouse.
232	3	2/3	1/3	? Metabolite in tumour mouse with large tumour. General fluorescence only in other tumour mouse.
307	10	0/10	0/10	

NOTE.—1 mouse died after 136 days with a tumour and persistent fluorescence at the site of injection and 1 after 165 days. These mice were not examined histologically or spectrographically.

DISCUSSION.

It is clear from the above results that with tricaprylin as solvent, benzpyrene persisted at the site of injection up to 6 months, at least in those mice which developed tumours. During the latent period a metabolite or metabolites also made their appearance at or near the site of injection. The absence of benzpyrene from extracts of two tumour-bearing mice killed 7 months after a single injection of benzpyrene does not preclude the possibility that the unchanged hydrocarbon was still present at the inception of the tumour process as it was in the other tumour-bearing animals killed after shorter periods.

Thus the results of this experiment accord well with our previous observations (Peacock and Beck, 1938) that when fluorescence remained at the site of injection in mice for 5 months or more there was a high incidence of sarcoma, whereas when no detectable fluorescence was present after 3 months, the sarcoma incidence was low. Furthermore, Weil-Malherbe (1946*b*) used a 5 per cent aqueous solution of tetramethyluric acid as a solvent for benzpyrene, and injected 0·3 ml., equivalent to 0·2 mg. benzpyrene, in mice. He found that under these conditions fluorescence was absent from the site of injection after 2 weeks, and only one out of 20 mice developed a tumour. One of us (S. B.) observed that an aqueous soap solution of benzpyrene is also quickly absorbed from the site of injection in mice, and no tumours have been found amongst 22 mice that survived for 6 months after injection, equivalent to 0·1 mg. of benzpyrene.

The method of examination of mice used by Dickens and Weil-Malherbe differed from ours. They extracted the whole animals and estimated the rate of elimination of benzpyrene in terms of the unchanged hydrocarbons in their extract. On this basis Dickens (1947) states that "the surprising result was obtained that the more rapid elimination of benzpyrene was associated with the higher carcinogenic activity, and slower elimination with lower activity."

The terms "rapid" or "slow" elimination require further definition. In fact the graphs published by Dickens and Weil-Malherbe (1946*b*) show, in the case of tricaprylin alone, and of tricaprylin plus phospholipids as solvents for benzpyrene, a consistent rate of elimination of the hydrocarbon from the site of injection, but not to the point of extinction within 85 days and 100 days respectively. Extrapolation of the tricaprylin graph would indicate total elimination in the region of 26 weeks. As 10 out of 26 mice had tumours by the 20th week, it seems reasonable to suppose that some benzpyrene was present in these mice throughout the latent period of carcinogenesis. This would correspond closely with our own experience. In the graph illustrating the course of events following injection of benzpyrene dissolved in tricaprylin plus 3 per cent cholesterol, Dickens and Weil-Malherbe (1946*b*) show a considerable variation in the persistence of benzpyrene in different mice. Thus their final observation on the 78th day showed no benzpyrene, but in the mouse observed on the 71st day about one-twelfth of the initial amount of 300 μg. benzpyrene was present, and some benzpyrene was demonstrated in all the other mice examined during the course of their experiment up to that date. As no tumours were recorded before about the 9th week of their experiment, there is no positive evidence about the persistence or absence of benzpyrene at the site of injection in their tumour-bearing mice. Our own repetition of their experiment, using tricaprylin as solvent, showed that benzpyrene was demonstrable in most of the tumour-bearing

mice, and must therefore have been present throughout the latent period of carcinogenesis.

While it seems certain that partition of benzpyrene between the solvent used as vehicle and the body fluids must be largely responsible for the rate of elimination of benzpyrene, the local tissue reaction to different solvents may also help to determine the local response. The further factor of local metabolism of benzpyrene may be concerned in the mechanism of carcinogenesis, but the evidence for the presence of metabolites at the site of injection of benzpyrene is less secure than the evidence for the persistence of the parent hydrocarbon.

The crucial question is the nature and duration of the contact between the carcinogen and the susceptible cell necessary to ensure carcinogenesis. Unfortunately we have no means of determining the actual latent period between exposure of a susceptible cell to a carcinogen and its conversion to malignancy, but the clinical latent period from the first exposure to the carcinogen until a palpable tumour is recognizable must exceed the actual latent period by an unknown amount. Moreover, we cannot estimate the proportion of susceptible cells at a given site of injection actually in contact with the injected carcinogen. The only practical way to ensure a statistically significant yield of tumours is to expose an excess of cells to an excess of carcinogen for an excessive time. Under such conditions the injected carcinogen forms a depot from which it is gradually absorbed. There is some evidence (Peacock and Beck, 1938) that sarcoma is most likely to arise, not at the point of maximum concentration of the carcinogen, but at some point along the route of its absorption, where optimum conditions occur. The cells in the path of absorption may thus be analogous to the epithelial cells of the skin exposed to repeated painting with a carcinogen, where the incidence of tumours varies directly as the length of exposure up to a maximal response.

The evidence suggests that carcinogenesis depends on many factors, only a few of which are under our control, but that, within the latent period for mice of 3 to 6 months, the chance of inducing a tumour varies directly as the persistence of benzpyrene at the site of injection.

After our experiments were completed, Strait, Hrenoff and de Ome (1948) published the results of experiments on the influence of solvents on the effectiveness of carcinogenic agents. They established that for benzpyrene the partition coefficient between human sera and various lipoid solvents was directly related to the carcinogenic response of mice injected with benzpyrene dissolved in those solvents. Solvents that readily parted with benzpyrene *in vitro* gave fewer tumours when used as vehicles for injection of benzpyrene in mice. Their conclusions support the conception that there is a significant relationship between the retention at the site of injection of benzpyrene and the ultimate carcinogenic response.

Whatever may be the essential mechanism of conversion of a normal cell to a malignant one, it seems certain that some local consumption of energy by some part of the cell must be involved in the transformation. It may well be that the liberation of energy at the optimum time in the life-cycle of a susceptible cell is the essential carcinogenic stimulus, and this may be provided by the metabolism of a carcinogen. In this event neither the parent hydrocarbon nor its metabolites, but the transition from one to the other, may provide the essential carcinogenic stimulus. The possible mechanism of such a stimulus was recently discussed by

one of us (Anderson, 1947*b*). We had hoped to obtain from the present series of experiments enough local metabolite of benzpyrene to test its possible carcinogenicity by injection into other mice; but the amounts obtained were barely enough for spectrographic analysis. Until a method of identifying and synthesising the metabolites of benzpyrene is devised, it seems unlikely that enough material will become available for this crucial test. In the absence of such evidence it appears that the presence of the parent hydrocarbon throughout the latent period of carcinogenesis is necessary for a high yield of local sarcoma under the conditions of our experiment.

SUMMARY.

1. The evidence that solvents profoundly modify the local action of carcinogenic hydrocarbons is briefly reviewed.

2. Benzpyrene dissolved in tricaprylin is shown to remain at the site of injection in progressively diminishing amounts throughout the latent period of sarcoma induction in stock mice.

3. Towards the end of the latent period evidence of the presence of a metabolite is found at or near the site of injection.

4. No evidence is yet available to show whether benzpyrene acts as such or through its metabolites to induce sarcoma.

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3:4-BENZPYRENE AS AN –SH INHIBITOR.

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THE question of the involvement of the polycyclic hydrocarbons with sulphydryl groups during the process of carcinogenesis has been under discussion for a long time. Certain indirect evidence exists and has been reviewed by Crabtree (1947), who concludes that the available evidence does not warrant any decision as to whether the involvement is direct or not.

In other directions Mueller and Rusch (1943) could obtain no effect upon the activity of urease—this being dependent upon the integrity of the –SH groups—with benzpyrene solution. On the other hand Rondoni and Bassi (1948) have found benzpyrene to inhibit the papainaise digestion of gelatin and also the cathepsins of rat sarcoma and horse liver. The non-carcinogenic hydrocarbons, phenanthrene, anthracene and pyrene, had no action. Calcutt and Newhouse (1948) also found that the photodynamic action of benzpyrene was inhibited by cysteine hydrochloride.

Further work (Calcutt, 1948) showed that certain other –SH-containing compounds were also effective as inhibitors of photodynamic action. In the attempt to find the mechanism of this action it was found that after incubation of a colloidal suspension of benzpyrene in a solution of cysteine hydrochloride the characteristic yellow-green fluorescence colour of the benzpyrene changed to pale blue. Preliminary attempts to isolate this blue fluorescing compound were unsuccessful, but it was found that the mixture no longer gave a positive test with sodium nitroprusside. This implied the inhibition of the –SH group of the cysteine and formed the starting-point for further work.

The inhibition of –SH groups.

Using colloidal benzpyrene in distilled water, tests were carried out with a series of compounds. In each case the technique was the same. Equal amounts of a solution of the sulphydryl-containing compound were placed in each of two tubes. To one was added some benzpyrene colloid, and to the other an equivalent amount of distilled water. Both were then incubated, and samples from each were tested at intervals with nitroprusside. In this way it was found that inhibition of the –SH of cysteine hydrochloride, thiomalic acid, thioglycollic acid and 2-mercaptoethanol occurred in the tubes containing benzpyrene. In each case the control tube continued to give a positive reaction with the nitroprusside. The inhibition of the –SH appeared to run parallel with a change in fluorescence

colour of the benzpyrene from yellow-green to pale blue. There was no apparent fluorescence in the water which acted as the medium.

In the above instances the reaction mixture was in each case acid. So the investigation was extended to cover a greater pH range. Using cysteine, sodium thiomalate, sodium thioglycollate solutions to which varying amounts of sodium hydroxide solutions had been added, it was found that inhibition occurred as before. With slightly alkaline mixtures, however, a small amount of blue fluorescing compound was obtained in solution.

For all the cases mentioned above incubation has to be continued for upwards of 1½ hours at a temperature of 65° C. before any inhibition became apparent. At lower temperatures the action was very slow, and at room temperature—25° C. —there appeared to be no effect whatever.

Preliminary trials having indicated that caffeine solutions had little effect upon –SH as detected by nitroprusside, some of the previous experiments were repeated, using a solution of benzpyrene in a 3 per cent caffeine solution. Inhibition of –SH was achieved as before, but this time much more rapidly—30 minutes at 65° C.

Isolation of the reaction products.

Under the conditions so far investigated the yield of the pale blue fluorescing product of the reactions has only been very small. Nevertheless it has been found possible to isolate products from both the alkaline and acid reaction mixtures. The methods used and found suitable were those developed by Weigert and Mottram (1946) and Weigert (1948) for the separation of benzpyrene metabolites as found in animals. In this way small yields of solutions of apparently pure substances were obtained, the final purifications being by repeated chromatography.

Properties of the reaction products.

To date insufficient material has been available for chemical analysis, but certain general properties are apparent. From the alkaline reaction mixture a water soluble compound was derived. This is soluble in organic reagents, strongly adsorbed to silica, strongly adsorbed to alumina, fluoresces a whitish-blue colour in ultra-violet light. On addition of dilute acid it changes to a blue fluorescing compound which is soluble in organic reagents, insoluble in water but soluble in dilute alkali.

From the acid reaction mixture a compound has been separated which is soluble in organic reagents, soluble in dilute alkali, insoluble in water, adsorbed strongly to alumina and is weakly adsorbed to silica, moving down slowly as elution is continued.

Thus in their physical properties these compounds show a remarkable similarity to those separated from animals and designated by Weigert and Mottram (1946) as BpX_1, BpF_1 and BpF_2, this latter apparently being identical with the compound characterized by Berenblum, Crowfoot, Holiday and Schoental (1943) as 8.OH Benzpyrene.

Examination of the fluorescence spectra of the derived compounds offers further interest. That of the water soluble compound is found to be comparable

to that of BpX_1, whilst the alkali soluble product derived by acidification gives a spectrum similar to that of BpF_1. In this connection it must be recalled that Weigert and Mottram (1946) found that acid hydrolysis of BpX_1 gave rise to BpF_1.

The spectrum of the alkali soluble product from the acid reaction mixture resembles that of BpF_2 (8.OH.Bp). Furthermore it is found that continued

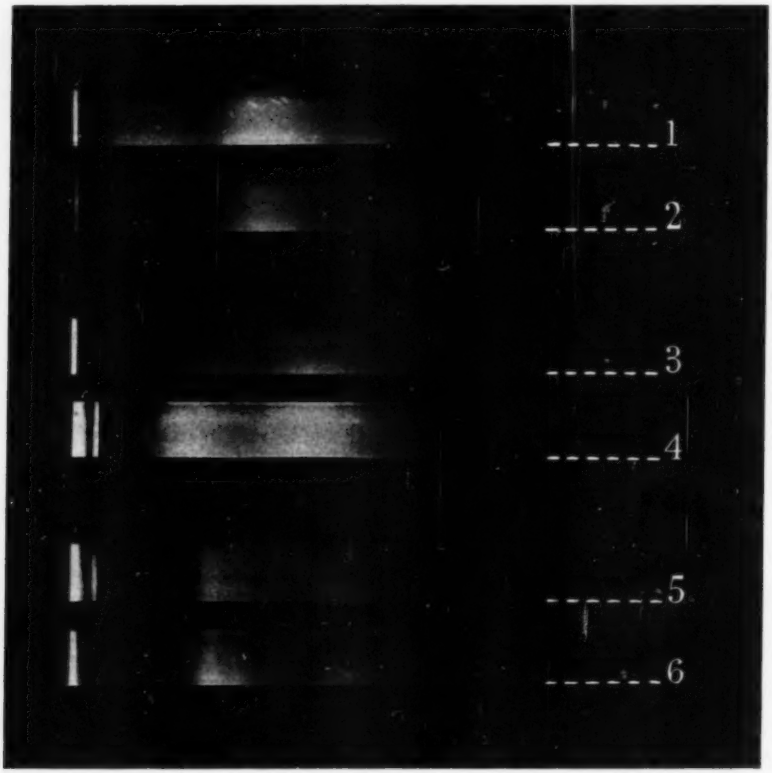

FIG. 1.—Fluorescence spectra of benzpyrene derivatives: (1) Benzpyrene X_1 from mouse. (2) Water soluble compound from alkaline reaction mixture. (3) Benzpyrene F_1 from mouse. (4) Alkali soluble product from acidification of (2). (5) 8.OH benzpyrene from mouse. (6) Alkali soluble product from acid reaction mixture.

acid hydrolysis of the compound derived from the water soluble product gives rise to a further compound, which from its general behaviour and fluorescence spectrum appears to be identical with the product derived from the acid reaction.

For purposes of comparison the fluorescence spectra of the compounds obtained as above are shown together with those of BpX_1, BpF_1 and BpF_2 in Fig. 1.

DISCUSSION.

The work described above indicates that 3 : 4-benzpyrene can engage in direct chemical action with a variety of compounds containing sulphydryl. Furthermore the evidence suggests a considerable parallelism, even if not identity, with the course of events after the introduction of benzpyrene into the animal body. Whilst the apparent identity of the fluorescence spectra does not imply strict chemical identity, it does mean the very close relationship of the substances concerned.

From this it is reasonable to suggest that the interaction of benzpyrene with a –SH containing compound gives rise to a substance of the BpX_1 type. This, however, is in the presence of acid successively broken to compounds of BpF_1 and BpF_2 type. It has already been suggested by Crabtree (1947) that the detoxication of benzpyrene is by way of –SH compounds. In view of the evidence presented above this must now be regarded as being most probable.

Crabtree (1940, 1944, 1945) has as a result of his work with –SH inhibitors suggested the involvement of sulphur in carcinogenesis caused by the polycyclic hydrocarbons. This is further reinforced by the recent work of Lusky, Braun and Woodard (1947), who inhibited the carcinogenic action of the benzpyrene by application of- B.A.L. (2:3-dimercaptopropanol). Additionally, evidence has been put forward by Weigert, Calcutt and Powell (1947) to the effect that detoxication occurs at the site of carcinogenesis. Now in view of the above evidence it appears most likely that detoxication and carcinogenesis both occur together, and that both processes are intimately concerned with sulphydryl.

Further evidence is required to reinforce the views expressed above, and to this end an investigation of a series of carcinogenic and non-carcinogenic hydrocarbons has been started whilst work on the chemistry of the reactions described is continuing. So far the data available indicates that other carcinogenic hydrocarbons react with –SH containing compounds in a fashion similar to benzpyrene, but that the non-carcinogenic ones show no similar activity. Full details will be published as soon as possible.

It is futile to speculate further on the role of sulphydryl in carcinogenesis, but in conclusion it is perhaps interesting to recall that Potter (1943) has postulated carcinogenesis as arising by production of abnormal protein ; Rondoni (1948) suggests the action of carcinogenic agents on nucleo- or liponucleo-protein, whilst Powell (1946, 1947) has put forward a hypothesis involving cytoskeletal protein with particular reference to thiol groups. In any case, sulphydryl is inextricably involved in discussions of proteins.

SUMMARY.

An account is given of the conditions under which 3:4-benzpyrene is found to inhibit the –SH groups of cysteine hydrochlroide, thiomalic acid, thioglycollic acid and 2-mercaptoethanol. The isolation and further examination of reaction products is described. On the basis of physical properties and fluorescence spectra these products are shown to be similar to the benzpyrene metabolites obtained from animals. The relationship of the above findings to carcinogenesis and detoxication are discussed.

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SURVEY OF PAPERS.

VERSLUYS has made a statistical analysis of the Registrar-General's records of deaths from carcinoma in the Netherlands during the years 1931 to 1935 to find what influence trade or profession has on the origin and the localization of carcinoma. He presents numerous detailed tables but considers that conclusions will be justifiable only when sufficient information from different countries is available (p. 161).

LEES AND PARK have investigated the question whether the malignancy of cancer is greater in relatively younger people by comparing the histological appearances of carcinomas from patients aged 30 years or less with those from patients of older age groups. They have found no significant differences except doubtfully in carcinoma of the lung (p. 186).

CAMPBELL has investigated a hitherto unrecorded neoplastic disease affecting the liver of the female Khaki Campbell duck and concludes that, while an associated transmissible factor cannot be excluded, there is evidence that a genetic factor may probably be involved in its aetiology (p. 198).

HORNING describes the early stages of tumour production in subcutaneous grafts of prostatic epithelium impregnated with 20-methylcholanthrene in the mouse. Of the tumours induced in this way, the glandular carcinomas regress when transplanted to castrated male mice and respond to treatmeut with diethylstilboestrol, whereas the squamous cell tumours show no appreciable response (p. 211).

FOULDS has demonstrated mammary tumour agent in one of his hormone-responsive transplantable tumours which originated in hybrid mice obtained by mating females of the low mammary tumour strain C57 blacks with males of the high mammary tumour strain R3. He also finds a high tumour incidence in the progeny of two tumour-bearing female hybrids and a low incidence in the progeny of one tumour-free female hybrid. He attributes his results, provisionally, to the erratic transmission of tumour agent by the male parents (p. 230).

FOULDS finds that the hormone-responsiveness of his transplanted tumours is not correlated with absence of the milk agent, that hormones regulate the activities of some tumours, and that responsiveness to hormones changes during serial transplantation (p. 240).

DMOCHOWSKI has shown that the milk factor is present in the cells of the 42nd serial transplant of a mammary tumour induced by the milk factor. He confirms that the genetic constitution of the host is responsible for the successful transplantation of tumours induced by the milk factor, and not the presence or absence of the factor in the host (p. 246).

CRAIGIE describes a pressure mincer for the rapid reduction of tumour tissue to a suspension of fine fragments and separated cells (p. 249).

CRAIGIE describes an apparatus for drying single samples of tumour suspension rapidly at low temperature (p. 250).

IDA MANN finds that minced mouse embryo tissue lives and grows when implanted under the skin of mice of the same inbred strain and growth is prevented if the embryo mince is previously frozen to − 79° C. for 1 to 24 hours or treated with glycerol and stored at 4° C. (p. 255).

GYE, BEGG, IDA MANN AND CRAIGIE find that minced tumour tissue from both chemically induced and sporadic sarcomas of inbred mice retains its power to start fresh tumours after prolonged storage at − 79° C. and even when it is dried after such storage. Some mouse sarcomas can be propagated by dried tissue without preliminary freezing. The authors conclude that the continuing course of these tumours is probably a virus (p. 259).

CRAIGIE has investigated the conditions necessary for the preservation of mouse tumour suspensions in the frozen state. The degree of survival of activity in dextrose preparations is adequate for experimental work requiring the use of transplantable tumours and may offer a more convenient source of material than fresh tumour tissue (p. 268).

BULLOUGH has shown that starvation or a restricted diet has a powerful effect in depressing epidermal mitotic activity in the mouse. This observation may explain previous findings that diet restriction greatly reduces the incidence of spontaneous and induced tumours (p. 275).

BIELSCHOWSKY AND BULLOUGH find that the yield of skin tumours induced in mice by a single application of a carcinogenic hydrocarbon, followed by repeated painting with croton oil, is the same whether or not mitotic activity in the epidermis is suppressed by starvation during the period of action of the carcinogen (p. 282).

CORNELIA HOCH-LIGETI has found pancreatic tumours in 3 rats which failed to develop liver tumours after the oral administration of *p*-dimethylaminoazobenzene for 17 months. In 2 of the rats the liver was protected by the addition of fresh milk to the diet. The effect of diet on the localization of tumours is discussed (p. 285).

ANDREE PEACOCK AND PEACOCK discuss the failure of their protracted attempts to induce epithelial tumours in the skin of fowls. Repeated injection of 2-acetylaminofluorene into the crop has been followed by ulcerated squamous carcinoma of the wall of the crop in 2 fowls (p. 289).

PEACOCK, BECK AND ANDERSON find that benzpyrene dissolved in tricaprylin remains at the site of subcutaneous injection in mice in progressively diminishing amounts throughout the latent period of sarcoma induction. Towards the end of the latent period there is evidence of the presence of a metabolite of benzpyrene (p. 296).

CALCUTT gives an account of the conditions under which benzpyrene inhibits the –SH groups of certain compounds and shows that the reaction products are similar to the benzpyrene metabolites obtained in animal experiments (p. 306).